



UNITED STATES PATENT AND TRADEMARK OFFICE

UNDER SECRETARY OF COMMERCE FOR INTELLECTUAL PROPERTY AND  
DIRECTOR OF THE UNITED STATES PATENT AND TRADEMARK OFFICE  
WASHINGTON, DC 20231  
www.uspto.gov

DAVID L. PARKER,  
FULBRIGHT & JAWORKI L.L.P.  
600 CONGRESS AVE.  
SUITE 2400  
AUSTIN, TX 78701

Paper No: 31  
Appeal No: 2004-1392  
Appellant: SASTRY, JAGANNADHA K.  
Application: 08/869,386

MAILED

MAY 19 2004

PAT. & T.M. OFFICE  
BOARD OF PATENT APPEALS  
AND INTERFERENCES

## Board of Patent Appeals and Interferences Docketing Notice

Application 08/869,386 was received from the Technology Center at the Board on April 23, 2004 and has been assigned Appeal No: 2004-1392.

A review of the file indicates that the following documents have been filed by appellant:

Appeal Brief filed on: March 31, 2000  
Reply Brief filed on: None  
Request for Hearing filed on: June 29, 2000

In all future communications regarding this appeal, please include both the application number and the appeal number.

The mailing address for the Board is:

**BOARD OF PATENT APPEALS AND INTERFERENCES  
UNITED STATES PATENT AND TRADEMARK OFFICE  
P.O. BOX 1450  
ALEXANDRIA, VIRGINIA 22313-1450**

The facsimile number of the Board is 703-308-7952. Because of the heightened security in the Washington D.C. area, facsimile communications are recommended. Telephone inquiries can be made by calling 703-308-9797 and should be directed to a Program and Resource Administrator.

By order of the Board of Patent Appeals and Interferences



# UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE  
United States Patent and Trademark Office  
Address: COMMISSIONER FOR PATENTS  
P.O. Box 1450  
Alexandria, Virginia 22313-1450  
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
08/869,386	06/05/1997	JAGANNADHA K. SASTRY	UTXC:538/HYL	5686

7590 01/14/2004

DAVID L. PARKER  
FULBRIGHT & JAWORSKI L.L.P.  
600 CONGRESS AVENUE, SUITE 2400  
AUSTIN, TX 78701

EXAMINER

LE, EMILY M

ART UNIT	PAPER NUMBER
----------	--------------

1648

30

DATE MAILED: 01/14/2004

Please find below and/or attached an Office communication concerning this application or proceeding.



## UNITED STATES DEPARTMENT OF COMMERCE

## U.S. Patent and Trademark Office

Address: COMMISSIONER FOR PATENTS

P.O. Box 1450

Alexandria, Virginia 22313-1450

APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
---------------------------------	-------------	---	---------------------

EXAMINER
----------

ART UNIT	PAPER
----------	-------

30

DATE MAILED:

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner for Patents

The Art Unit location of your application in the USPTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Art Unit 1648, Examiner Emily Le.

Paper No. 27, Request for Remand and Reconsideration, and paper No. 28, Declaration of Pramod N. Nehete will not be considered. This is in pursuant to 37 CFR 1.195, which states: Affidavits, declarations, or exhibits submitted after the case has been appealed will not be admitted without showing of good and sufficient reasons why they were not earlier present. The papers submitted lacks good and sufficient reasons as to why it was not presented prior to appeal.

*James C. House*  
JAMES HOUSEL 1/12/04  
SUPERVISORY PATENT EXAMINER  
TECHNOLOGY CENTER 1600

*Emily Le* 12/30/03  
Emily Le  
AU 1648  
(703) 305 4452

**NAME** Pramod N. Nehete, Ph.D.

**PRESENT TITLE AND AFFILIATION**

**Primary Appointment**

Assistant Professor

The University of Texas M D Anderson Cancer Center

Science Park – Department of Veterinary Sciences, Bastrop, TX

**Dual/Joint Appointment**

N/A

**CITIZENSHIP AND VISA STATUS (if appropriate)**

USA

**HOME ADDRESS (current)**

Bastrop, TX 78602

**OFFICE ADDRESS (current)**

650 Cool Water Drive, Bastrop, TX 78602

**EDUCATION**

**Degree-Granting Education**

Vikram University, Ujjain, India, B.S., 1975, Biology/Chemistry

G.B. Pant Univ. of Agriculture and Technology, Pantnager, India, M.S., 1980,

Biochemistry

Dept. of Chemistry, University of Poona, Pune, India, Ph.D., 1989, Biochemistry

**Postgraduate Training**

Graduate Research Assistant, Dept of Biochemistry, G.B. Pant University of Agriculture and Technology, Pantnager, India, 1975-1977.

Research Scientist, Sarabhai Research Center, Baroda, India, Supervisor: Dr. RM Kothari, 1977-1990

Project Investigator, Dept. of Molecular Pathology, UT M D Anderson Cancer Center, Houston, TX, Supervisors: Dr. RB Arlinghaus and Dr KJ Sastry, 1990-1993.

**CREDENTIALS**

**Board Certification**

N/A

**Licensure(s)**

N/A

Active

Inactive

**EXPERIENCE/SERVICE**

**Academic Appointments**

Research Associate, The University of Texas M D Anderson Cancer Center, Department of Molecular Pathology, Houston, TX, 9/1993-8/1994.

Research Associate, The University of Texas M D Anderson Cancer Center, Science Park–Department of Veterinary Sciences, Bastrop, TX, 9/1994–12/1995.

Assistant Professor, The University of Texas M D Anderson Cancer Center, Science Park–Department of Veterinary Sciences, Bastrop, TX, 1/1996-Present

**Academic Administrative Appointments/Responsibilities**

N/A

**Institutional Committee Activities**

N/A

**Other Appointments/Responsibilities**

N/A

**Consultantships**

N/A

**Military or Other Governmental Service**

N/A

**HONORS AND AWARDS**

Awarded national scholarship for graduate studies.

Awarded the Center for Scientific and Industrial Research (CSIR) fellowship for post-graduate studies.

**RESEARCH**

**Grants and Contracts**

Project Investigator, HIV Envelope Peptide-Based Vaccine in SHIV-Rhesus Model, PI KJ Sastry, NIH/NIAID #RO A146969-02, 4/01/2000-3/31/2002, \$340,000 annual budget.

Project Investigator, HPV Specific Cellular Immunity in CIN Patients: A Prospective Study, PI KJ Sastry, DHHS/NIH National Cancer Institute 5R01CA77378-03, 8/1/199-5/31/2004, \$352,792 annual budget.

Co-Principal Investigator, Studies on Therapeutic Potentials of HIV Synthetic Peptides: Development of a Peptidomimetic Form of R15K, BioQuest/Biokeys, Inc, Sponsored Research Agreement #96-006/A1, PI KJ Sastry, Open, \$267,837.

Co-Principal Investigator, Studies on Therapeutic Potentials of HIV Synthetic Peptides, BioQuest/Biokeys, Inc, Sponsored Research Agreement #96-006, PI KJ Sastry, Open, \$755,619.

Co-Principal Investigator, Studies on Therapeutic Potentials of HIV Synthetic Peptides: Clinical and Pre-clinical Studies, BioQuest/Biokeys, Inc, Sponsored Research Agreement #96-006/A2 (SR96-06B), PI KJ Sastry, Open, \$126,916

**Funded Protocols**

N/A

**Patents Granted and Pending**

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Methods and compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Australian Patent No. 666160, 5/20/1996.

VA Dwyer, KJ Sastry, RB Arlinghaus, PN Nehete, CD4 peptides for binding to viral envelope proteins, US SN: 08/115,171, 6/17/1996.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Use of a composition comprising a peptide for the inhibition of HIV. European Patent 9911207.2107, 8/17/1999.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-Lymphocyte responses against viruses. US SN 08/860,386, European Patent: 0671947 B1. 3/8/2000.

**Pending:**

KJ Sastry, R Arlinghaus, C Platsoucas, PN Nehete. Compositions and methods for eliciting immune or anti-infective response (a) enhancement and systemic spread of virus-specific CTL responses mediated by mixtures of helper T-cell and CTL inducing peptides. (SN 08/869,386) filed 9/16/1992

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Inhibition of HIV type-1 infection of human cells by synthetic peptides from gp120. A divisional patent application is pending in the USA, European Patent issued and an International Patent is pending.

KJ Sastry, RB Arlinghaus, PN Nehete, HIV-specific T-cell induction (SN 60/108,563)

KJ Sastry, VA Dwyer, RB Arlinghaus, PN Nehete, Peptides for inhibiting the infection of target cells by lentiviruses.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Swedish Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Spanish Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Italian Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Dutch Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, French Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Greek Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Portuguese Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Danish Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Irish Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Austrian Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Swiss Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, Belgian Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, German Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, CD Platsoucas, PN Nehete, Compositions for eliciting cytotoxic T-lymphocyte responses against viruses, British Patent application based on European Patent Application No. 9300770.4 from PCT/US 92/10378 and US SN 07/800,932 and 07/945,865.

KJ Sastry, RB Arlinghaus, PN Nehete, HIV-Specific T-Cell Induction. Canadian Counterpart Patent Application Based on International Patent Application No. PCT/US99/27185 and US SN 60/108,563 and 60/115,175, filed 5/15/2001.

KJ Sastry, RB Arlinghaus, PN Nehete, HIV-Specific T-Cell Induction European Patent Application No. 99865820.6-1219, Serial No. 1131633, Based on International Patent Application No. PCT/US99/27185 and US SN 60/108,563 and 60/115,175, published 9/12/2001.

KJ Sastry, RB Arlinghaus, PN Nehete, HIV-Specific T-Cell Induction Japanese Patent Application No. 2000-582054 Based on International Patent Application No. PCT/US99/27185 and US SN 60/108,563 and 60/115,175.

Invention Disclosure: KJ Sastry and PN Nehete, Reagents and methods for adjuvant-free vaccines against infectious diseases and cancer.

**Grant Reviewer/Service on NIH/Other Study Sections**

N/A

## **PUBLICATIONS**

### **a. Articles in Peer-Reviewed Journals**

Nehete PN, Shah VD, Kothari RM. Profiles of alkaline protease production as a function of composition of the slant, age, transfer and isolate number of physiological sature of culture. Biotechnol Letters 7:413-8, 1985.

Nehete PN, Shah VD, Kothari RM. Isolation of higher yielded of alkaline protease. Enzyme and microbial Technol 8:370-2, 1986,

Shah DN, Shah VD, Nehete PN, Kothari RM. Isolation of *B locheniformic* mutant for stable production profiles of alkaline protease. Biotechnol Letters 8:103-6, 1986.

Nehete PN, Kothari RM, Shankar V. Immobilization of amyloglucosidase on an anion exchanger: I. Preparation and properties. Food and Technol 1:107-16, 1987.

Nehete PN, Hegde MV, Reddy LG, Shankar V. Immobilization of amyloglucosidaes on polystyrene anion exchange resin. II. Kinetics and stabilities. Biotechnol Letters 9:651-6, 1987.

Shah NK, Shah DN, Upadhyay CM, Nehete PN, Kothari RM. An economical, upgraded, stabilized and efficient preparation of amyloglucosidase. J Biotechnol 10:267-76, 1989.

Shah NK, Shah VD, Nehete PN, Kothari RM. Isolation of a stable and high yielding alpha-amylase variant of *B. subtilis*. J. Biotechnol 11:67-74, 1989.

Upadhyay CM, Nehete PN, Kothari RM. Studies on the production of lipase from *R. oligosporous*. Biotechnol Letters 11:793-6, 1989.

Shah NK, Upadhyay CM, Nehete PN, Kothari RM, Hegde MR. A upgraded, stabilized and efficient preparation of alpha-amylase. J. Biotechnol 16:97-108, 1990.

Nehete PN, Shah NK, Kothari RM. Recycling of mother liquor of sorbose and glucose for hexitol production. Resources, Conserv & Recycling 5:81-7, 1991.

Upadhyay CM, Nehete PN, Shah DN, Shah NK, Shankar V, Kothari RM. Alternate economical starchy substrates for the production of 70% sorbitol. Starch 43(3):107-13, 1991.

Sastry KJ, Nehete PN, Kan S, *et al*. Membrane-permeable dideoxyuridine 5"-monophosphate analogue inhibits human immunodeficiency virus infection, Mol Pharmacol 41(3):441-5, 1992.

Sastry KJ, Nehete PN, Venkatnarayanan S, Morkowski J, Platsoucas CD, Arlinghaus RB. Rapid *in vivo* induction of HIV-specific CD8<sup>+</sup> cytotoxic T lymphocytes by a 15-amino acid unmodified free peptide from the immunodominant V3-loop of gp120. Virology 188(2):502-9, 1992.

Nehete PN, *et al*. Immobilization of amyloglucosidase on polystyrene anion exchange resin: III Product analysis. Food Biotechnol 6:127-34, 1992.

Nehete PN, Shsh NK, Ramamurthy V, Kothari RM. An optimized protocol for the production of high purity maltose. World J Microbiol & Biotechnol, 1992.

Shankar V, Nehete PN, Kothari RM. Immobilization of amyloglucosidase [Review]. Indian J Biochem & Biophysics 30(1):62-70, 1993.

Nehete PN, Satterfield WC, Matherne CM, Arlinghaus RB, Sastry KJ. Induction of human immunodeficiency virus-specific T cell responses in rhesus monkeys by synthetic peptides from gp160. AIDS Res and Human Retroviruses 9(3):235-40, 1993.

Nehete PN, Arlinghaus RM, Sastry KJ. Inhibition of human immunodeficiency virus type 1 infection and syncytium formation in human cells by V3 loop synthetic peptides from gp120. J Virol 67(11):6841-6, 1993.



Ramamurthy V, Upadhyay Cm, Nehete PN, *et al.* Industrial significant enzymes: Strategy for R&D, semi-commercial production, upgradation, stabilization and applications [Review]. Hindustan Antibiotics Bulletin 35(1-2):43-76, 1993.

Nehete PN, Arlinghaus RB, Sastry KJ. Use of helper T cell-inducing peptides from conserved regions in HIV-1 *env* in a noncovalent mixture with a CTL-inducing V3-loop peptide for *in vivo* induction of long-lasting systemic CTL response. Vial Immunol 7(4):189-97, 1994.

Nehete PN, Casement KS, Arlinghaus RB, Sastry KJ. Studies on *in vivo* induction of HIV-1 envelope-specific cytotoxic T lymphocytes by synthetic peptides from the V3 loop region of HIV-1 IIIB gp120. Cell Immunol 160(2):217-23, 1995.

Nehete PN, Murthy KK, Satterfield WC, Arlinghaus RB, Sastry KJ. Studies on V3-specific cross-reactive T-cell responses in chimpanzees chronically infected with HIV-1 IIIB. AIDS 9(6):567-72, 1995.

Casement KS, Nehete PN, Arlinghaus RB, Sastry KJ. Cross-reactive cytotoxic T lymphocytes induced by V3 loop synthetic peptides from different strains of human immunodeficiency virus type 1. Virology 211(1):261-7, 1995.

Sarkar AK, Tortolero-Luna G, Nehete PN, Arlinghaus RB, Mitchell MR, Sastry KJ. Studies on *in vivo* induction of cytotoxic T lymphocyte responses by synthetic peptides from E6 and E7 oncoproteins of human papillomavirus type 16. Viral Immunol 8(3):165-74, 1995.

Nehete PN, Johnson PC, Schapiro SJ, Arlinghaus RB, Sastry KJ. Cross-reactive T-cell proliferative responses to V3 peptides corresponding to different geographical HIV-1 isolates in HIV-seropositive individuals. J Clin Immunol 16(2):115-24, 1996.

Sastry KJ, Marin MC, Nehete PN, McConnell K, El-Naggar AK, McDonnell TJ. Expression of human immunodeficiency virus type 1 *tat* results in down-regulation of bcl-2 and induction of apoptosis in hematopoietic cells. Oncogene 13(3):487-93, 1996.

Schapiro SJ, Nehete PN, Perlman JE, Bloomsmith MA, Sastry KJ. Effects of dominance status and environmental enrichment on cell-mediated immunity in rhesus macaques. Appl Anim Behav Sci 56:319-32, 1998.

Oka T, Sastry KJ, Nehete P, *et al.* Evidence for specific immune response against P210 BCR-ABL in long-term remission CML patients treated with interferon. Leukemia 12:155-63, 1998.

Nehete PN, Schapiro SJ, Johnson PC, Murthy KK, Satterfield WC, Sastry KJ. A synthetic peptide from the first conserved region in the envelope protein gp160 is a strong T-cell epitope in HIV-infected chimpanzees and humans. Viral Immunity 11(3):147-158, 1998.

Nehete PN, Lewis DE, Tang DN, Pollack MS, Sastry KJ. Presence of HLA-C-restricted cytotoxic T-lymphocyte responses in long-term nonprogressors infected with human immunodeficiency virus. Viral Immunology 11(3):119-29, 1998.

Schapiro SJ, Nehete PN, Perlman JE, Sastry KJ. A comparison of cell-mediated immunity in rhesus macaques housed singly, in pairs, or in-groups. Appl Anim Behav Sci 68:67-84, 2000.

Dolbier CL, Cocke RR, Leiferman JA, *et al.* Differences in functional immune responses of high vs. low hardy healthy individuals, J Behav Med 24(3):219-29, 2001.

Sastry KJ, Nehete PN, Savary, CA. Impairment of antigen-specific cellular immune responses under simulated microgravity conditions. *In Vitro Anim Cell & Dev Biology* 37:203-8, 2001.

Nehete PN, Chitta S, Hossain MM, *et al.* Protection against chronic infection and AIDS by an HIV envelop peptide-cocktail vaccine in a pathogenic SHIV rhesus model. *Vaccine (In Press)*.

**b. Invited Articles**

Sastry KJ, Nehete PN, Casement K, Platsoucas CD, Arlinghaus RB. Rapid induction of virus-specific MHC-restricted CTLs with short synthetic peptides. In: *Vaccines 93*, RA Lerner, F Brown, HS Ginsberg (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 19-23, 1993.

Nehete PN, Arlinghaus RB, Sastry KJ, Satterfield WC, Matherne CM. HIV-specific T-cell responses in rhesus monkeys immunized with synthetic peptides from gp160. In: *Vaccines 93*, RA Lerner, F Brown, HS Ginsberg (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 91-4, 1993.

Sastry KJ, Nehete P, Casement K, Arlinghaus RB. Some synthetic peptides representing HIV-specific CTL epitopes fail to induce CTL responses *in vivo*: Implications for vaccine development. In: *Vaccines 94*, F Brown, R. Chanock, H Ginsberg (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 175-80, 1994.

Nehete PN, Arlinghaus RB, Sastry KJ. V3 loop synthetic peptides block infection and syncytium formation by HIV-1 In: *Vaccines 94*, F Brown, R. Chanock, H Ginsberg (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp.285-9, 1994.

Nehete PN, Chitta S, Hossain MM, Hill L, Bernacky B, Sastry KJ. Synthetic peptide-based HIV vaccine induces protective immunity in SHIV-rhesus model. In: *Peptides for the New Millenium*, GB Fields, JP Tam, G Barany (eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 706-7, 2000.

Hossain MM, Nehete PN, Chitta S, Sastry KJ. Synthetic peptide-based reagents for blocking the entry and inactivation of HIV. In: *Peptides for the New Millenium*, GB Fields, JP Tam, G Barany (eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 258-9, 2000.

Sastry, KJ, Nehete PN, Chitta S, *et al.* W. A conserved HIV envelope peptide cocktail vaccine provides efficient control of simian human immunodeficiency virus infection in rhesus monkeys. In: *Workshop on HIV/AIDS Vaccine Development*, National Agency for AIDS Research, Paris France, page 76, 2000.

Sastry KJ, Nehete PN, Gambhira R, Nehete B, Keeney TS. Synthetic peptide-based vaccine for induction of cell-mediated immunity against HIV and HPV, review article for publishing in second issue of the series *Virology* in book titled "*Recent Research Developments in Virology*, 3/01-Accepted for publication January 2002.

**c. Editorials**

N/A

**d. Other Articles**

N/A

**e. Abstracts (Last five years only)**

N/A

**f. Book Chapters**

N/A

**g. Books (edited and written)**

- N/A
- h. **Letters to the Editor**  
N/A
- i. **Manuals, Teaching Aids, Other Publications**  
N/A
- j. **Other**  
N/A

**EDITORIAL AND REVIEW ACTIVITIES****Editor/Service on Editorial Board(s)**

N/A

**Journal Reviewer**  
N/A

**TEACHING****Within Current Institution****Formal Teaching****Courses Taught**

N/A

**Training Programs**

N/A

**Other Educational Programs**

N/A

**Supervisory Teaching****Advisory Committees**

Member, Advisory Committee, Melissa West, 1998-2001.

**Supervisory Committees****Direct Supervision****Undergraduate and Allied Health Students**

Faculty, Dept. of Veterinary Sciences, Ben Winders, College Student, 240 hours,  
Summer 2000.

**Medical Students**

N/A

**Graduate Students****Residents and Fellows**

Supervisor, Dept. of Veterinary Sciences, Fasial Guhad DVM, PhD August 1999-  
May 2001

Supervisor, Dept. of Veterinary Sciences, Ratish Gambhir, BVSc, MVSc, May  
2000-Present

Supervisor, Dept. of Veterinary Sciences, Taletha Keeney, MS, May 2000-  
Present

Supervisor, Dept. of Veterinary Sciences, Bharti Nehete, BSc, September 2000-  
Present

**Outside of Current Institution****Organization of National or International Conferences/Symposia (Include chairing session)****Presentations at National or International Conferences****Invited**

Induction of HIV-specific T-cell responses in rhesus monkeys by synthetic peptides from gp160 by Nehete P, Satterfield W, Sharma V, Arlinghaus R, Sastry J.

Cold Spring Harbor Laboratory's Seminar on "Modern Approaches to New Vaccines Including Prevention of AIDS, Cold Spring Harbor, NY, September 16-20, 1992.

Synthetic peptides from the immunodominant V3 loop of gp120 inhibit infection of human cells by HIV-1 by Sastry KJ, Nehete P, Arlinghaus RB. Cold Spring Harbor

Laboratory's Seminar on "Modern Approaches to New Vaccines Including Prevention of AIDS, Cold Spring Harbor, NY, September 20-24, 1993.

V3 loop synthetic peptides block infection and syncytia formation by HIV-1 by Nehete PN, Arlinghaus RB, Sastry KJ. Cold Spring Harbor Laboratory's Seminar on "Modern Approaches to New Vaccines Including Prevention of AIDS, Cold Spring Harbor, NY, September 20-24, 1993.

Some synthetic peptides representing HIV-specific CTL epitopes fail to induce CTL responses *in vivo*: Implication for vaccine development by Sastry KJ, Casement K, Nehete PN, Arlinghaus RM. Cold Spring Harbor Laboratory's Seminar on "Modern Approaches to New Vaccines Including Prevention of AIDS, Cold Spring Harbor, NY, September 20-24, 1993.

Efficacy of a synthetic peptide –based vaccine in the SHIV rhesus model by Sastry KJ, Nehete P, Chitta S, Hossain MM, Hill L, Bernacky B. AVRC Workshop, Bethesda, MD, May 3-5, 1999.

Efficacy of a highly conserved HIV envelope peptide cocktail vaccine in the SHIV-rhesus model by Nehete PN, Chitta S, Hossain MM, Hill L, Arlinghaus RB, Sastry KJ. 2001 International Meeting of the Institute of Human Virology, Baltimore, MD, September 9-13, 2001.

HIV envelope peptide cocktail vaccine for selective priming of cell-mediated immunity and protection against pathogenic SHIV Challenge in Rhesus Macaques by Sastry, K, Nehete PN, Chitta S, Hossain MM, Hill L, Keeney T, Nehete B, Arlinghaus RB. 19<sup>th</sup> Annual Symposium on Nonhuman Primate Models for Aids, San Juan, Puerto Rico, November 7-10, 2001.

#### **Other, Including Scientific Exhibitions**

Poster presentation and co-author: A new strategy for chemotherapy of acquired immunodeficiency syndrome: Membrane-permeable dideoxy uridine monophosphate analogues as potent inhibitors of human immunodeficiency virus infection by Sastry KJ, Nehete PN, Khan S, Plunkett W, Arlinghaus RB and Farquhar DA. 8<sup>th</sup> Annual Meeting of Laboratory of Tumor Cell Biology, National Cancer Institute, NIH, Bethesda, MD September 1991.

Poster presentation co-author, Autocrine growth-factor activity in human neoplastic B-cells by Nehete PN, Sharma V, Sastry KJ, Arlinghaus RB, Sahasrabudhe CG. FASEB Journal 5(4):A541, 1991.

Poster presentation co-author, Rapid induction of HIV-specific cytotoxic T lymphocytes by an unmodified free peptide from the V3 loop of gp120 by Sastry J, Nehete P, Sharma V, Morkowski J, Platsoucas C, Arlinghaus R. Keystone Symposia on Molecular and Cellular Biology – Prevention and Treatment of AIDS, Keystone, CO, March 27-April 3, 1992.

Poster presentation co-author, Synthetic peptides from the immunodominant V3 loop of gp120 inhibit infection of human cells by HIV-1 by Sastry KJ, Nehete P, Arlinghaus RB. Cold Spring Harbor Laboratory's Meeting on "Modern Approaches to New Vaccines Including Prevention of AIDS", Cold Spring Harbor, NY, September 16-20, 1992.

Poster presentation co-author, Importance of determining specific CTL-inducing capacity of known CTL epitope: Application for vaccine development by Casement K, Nehete P, Bender B, *et al.* 45<sup>th</sup> Annual symposium on Fundamental Cancer

Research – Immunobiology of Cancer: Cellular and Molecular Mechanisms, UT M D Anderson Cancer Center, Houston, TX, October 20-23, 1992.

Poster presentation co-author, Induction of HIV specific T-Cell responses in rhesus monkeys by synthetic peptides from conserved regions of HIV *env* gp160 by Nehete P, Satterfield W, Arlinghaus R, Sastry J. 45<sup>th</sup> Annual symposium on Fundamental Cancer Research – Immunobiology of Cancer: Cellular and Molecular Mechanisms, UT M D-Anderson Cancer Center, Houston, TX, October 20-23, 1992.

Poster presentation co-author, Multifunctional synthetic peptides: Blockage of HIV infection, syncytia formation and induction HIV-specific CTL responses by Nehete P, Arlinghaus R, Sastry KJ, Casement K. Keystone Symposia on Molecular and Cellular Biology, Taos, NM, March 17-24, 1993.

Poster presentation co-author, Studies on the cross reactivity of HIV-1 V3 loop peptide induced cytotoxic T Lymphocytes by Casement K, Nehete P, Arlinghaus R, Sastry J. Cambridge Healthtech Institute's Conference on "Vaccines: New Technology and Applications", Washington, DC, March 22-24, 1993.

Poster presentation co-author, Inhibition of HIV-1 infection and syncytia formation by synthetic peptides from the V3 loop in gp120: Potential for AIDS therapy by Nehete P, Arlinghaus R, Sastry J. 93<sup>rd</sup> General Meeting of the American Society for Microbiology, Atlanta, GA, May 16-20, 1993.

Poster presentation co-author, V3 loop synthetic peptides block infection and syncytia formation by HIV-1 by Nehete PN, Arlinghaus RB, Sastry KJ. Cold Spring Harbor Laboratory's Meeting on "Modern Approaches to New Vaccines Including Prevention of AIDS:", Cold Spring Harbor, NY, September 20-24, 1993.

Poster presentation co-author, Synthetic HIV peptides for prophylaxis and treatment of HIV-induced acquired immunodeficiency syndrome by Nehete P, Casement K, Arlinghaus R, Sastry KJ. First International Conference on Engineered Vaccines for Cancer and AIDS, San Francisco, CA, September 30-October 2, 1993.

Poster presentation co-author, Blocking human immunodeficiency virus infection and cell to cell transmission by highly stable synthetic peptides by Nehete P, Sastry KJ, Casement K, Arlinghaus RB. Texas Triangle Meeting, Dallas, TX, March 18-19, 1994.

Poster presentation co-author, Protection of nude mice from HIV gp160 expressing tumors by adoptive transfer of HIV peptide-induced CTLs by Sastry KJ, Nehete P, Casement K, Arlinghaus RB. Texas Triangle Meeting, Dallas, TX, March 18-19, 1994.

Poster presentation co-author, Synthetic HIV peptides for prophylaxis and treatment of HIV-induced acquired immunodeficiency syndrome by Sastry KJ, Nehete P, Casement K, Arlinghaus RB. 16<sup>th</sup> International Congress of Biochemistry and Molecular Biology, New Delhi, India, September 19-22, 1994.

Poster presentation co-author, HIV-specific cell-mediated immunity in the absences of antiviral antibody response: Development of synthetic peptide-based reagents [abstract #90] by Sastry KJ, Nehete PN, Johnson PC, Murthy KK, Arlinghaus RB. 13<sup>th</sup> Annual Symposium on Nonhuman Primate Models for AIDS, Monterey, CA, November 5-8, 1995.

Poster presentation co-author, Effects of environmental enrichment on cellular immune function of rhesus macaques by Schapiro SJ, Nehete PN, Perlman JE, Sastry KJ. XVI<sup>th</sup> Congress of the International Primatological Society, Madison, WI, August 10-16, 1996.

Poster presentation co-author, Syncytium formation and/or infection by T-cell and microphage tropic HIV strains are inhibited by synthetic peptides from the V3-loop region via specific binding to cell surface by Sastry KJ, Nehete PN, Sarkar AK, Arlinghaus RB. Cambridge symposia: "New Insights in HIV Infection and Disease" Hilton Head, SC, October 10-13, 1996.

Poster presentation co-author, Cell surface binding and blocking HIV infection (T-cell and macrophage tropic strains) by V3 synthetic peptides by Sastry KJ, Nehete PN, Sarkar AK. 1997 Palm Springs Symposium on HIV/AIDS: "Foundations of HIV Therapy", Palm Springs, CA, March 13-15, 1997.

Poster presentation co-author, A post-CD4 binding step common to infection by T-cell- and macrophage-tropic HIV-1 strains involves cell surface interaction with the V4 region of viral gp120 by Nehete PN, Sarkar KA, Liu J, Arlinghaus RB, Sastry, KJ. Symposium of HIV-1 Infection, Mucosal Immunity & Pathogenesis, Natcher Conference Center, National Institutes of Health, Bethesda, MD, September 11-13, 1997.

Poster presentation co-author, HLA-C restricted cytotoxic T lymphocyte responses in long-term HIV<sup>+</sup> survivors by Nehete PN, Lewis DE, Tang DN, Pollack MS, Sastry KJ. 23<sup>rd</sup> Annual Meeting of the American Society for Histocompatibility and Immunogenetics, Atlanta, GA, October 14-19, 1997.

Poster presentation co-author, The relationship between resiliency, stress, symptoms of illness, and immune function by Stenhardt M, Dolbier C, Sastry KJ, *et al*. Psychology of Health, Immunity, and Disease Conference, Hilton Head, SC, December 1997.

Speaker at UT M. D. Anderson Cancer Center, Science Park – Department of Veterinary Sciences' Faculty Seminar Series: "Towards development of a synthetic peptide-based HIV vaccine" Bastrop, TX, April 16, 1998.

Poster presentation co-author, Development of novel reagents for HIV prophylaxis: Studies in multiple animal models and HIV+ long-term nonprogressors by Nehete PN, Lewis De, Murthy KK, Johnson PC, Sastry KJ. Cancer Vaccines '98 Conference, Bethesda, MD, April 27-28, 1998.

Poster presentation co-author, A novel HIV vaccine strategy involving the use of dendritic cells to present conserved HIV *env* peptides in SHIV-rhesus model by Sastry KJ Nehete P, Chitta S, Bernacky B, Hill, L. HIV Vaccine Development: Opportunities and Challenges, Keystone, CO, January 7-11, 1999.

Poster presentation co-author, Control of infection and pathology in SHIV-infected rhesus monkeys by synthetic peptide-based vaccine by Chitta S, Nehete P, Hossain M, Hill L, Bernacky B, Sastry J. Experimental Biology '99, Washington, DC, April 17-21, 1999.

Poster presentation co-author, Synthetic peptide-based HIV vaccine: Protective efficacy in the SHIV-rhesus model by Sastry KJ, Nehete PN, Chitta S, Hossain MM, Hill L, Bernacky B. A.V.P.S. 10<sup>th</sup> Symposium on HIV, Toulon, France, June 17-19, 1999.

Poster presentation and co-author, Synthetic peptide-based HIV vaccine induces protective immunity in SHIV-rhesus model by Nehete PN, Chitta S, Hossain MM, Hill L, Bernacky B, Sastry KJ. 16<sup>th</sup> American Peptide Symposium, St. Paul, MN, June 27-July 1, 1999.

Poster presentation and co-author, Synthetic peptide-based reagents for blocking the entry and interaction of HIV by Hossain MM, Nehete PM, Chitta S, Sastry KJ. 16<sup>th</sup> American Peptide symposium, St. Paul, MN, June 27-July 1, 1999.

Poster presentation and co-author, Synthetic peptide-based vaccine for controlling SHIV infection in rhesus monkeys by Sastry KJ, Nehete PM, Chitta S, Hossain MM, Hill L, Bernacky B. 17<sup>th</sup> Annual Symposium of Nonhuman Primate Models for AIDS. New Orleans, LA, October 6-9, 1999.

Poster presentation and co-author, Immunostimulatory characteristics of dendritic cells from rhesus monkeys by Chitta S, Nehete PM, Hossain MM, Sastry KJ. 17<sup>th</sup> Annual Symposium of Nonhuman Primate Models for AIDS, New Orleans, LA, October 6-9, 1999.

Poster presentation and co-author, Influence of psychosocial factors on immune status in rhesus monkeys: Implications for HIV research in non-human primates by Nehete PN, Schapiro SJ, Perlman JE, Sastry KJ. 17<sup>th</sup> Annual Symposium of Nonhuman Primate Models for AIDS, New Orleans, LA, October 6-9, 1999.

Poster presentation and co-author, A conserved HIV envelope peptide cocktail vaccine provided efficient control of Simian Human Immunodeficiency Virus infection in rhesus monkeys by Sastry KJ, Nehete PN, Chitta S, *et al.* Workshop on HIV/AIDS Vaccine Development, Pasteur Institute, Paris, France, May 5-6, 2000.

Poster presentation and co-author, HIV envelope peptide cocktail vaccine protects against chronic infection and AIDS in the SHIV-rhesus model by Nehete PN, Chitta S, Hossain MM, Hill, L, Arlinghaus RB, Sastry KJ. AIDS Vaccine 2001 Conference, Philadelphia, PA, September 5-8, 2001.

Poster presentation and co-author, A post-CD4-binding step involving the central V3-loop region is common to infection by X4 and R5 strains of HIV-1 by Nehete P, Hossain M, Fanti J, Sastry KJ. AIDS Vaccine 2001 Conference, Philadelphia, PA, September 5-8, 2001.

Poster presentation and co-author, Post-CD4 binding of HIV1 gp120 to host cell membrane glycosphingolipids through the V3 region is an important and common event for the entry of diverse HIV-1 strains by Vela E, Nehete, P, Houssain M, Yaki N, Fantini J, Sastry KJ, 19<sup>th</sup> Annual Symposium on Non-human Primate Models for Aids, San Juan, Puerto Rico, November 7-10, 2001.

Poster presentation and co-author, Improving the efficiency of ELISPOT analyses of cell-mediated immune responses in rhesus macaques in vaccine trials by Nehete PN, Gambhira R, Nehete B, Keeney, T, Lomad K, Chitta S, Sastry KJ. 19<sup>th</sup> Annual Symposium on Non-human Primate Models for Aids, San Juan, Puerto Rico, November 7-10, 2001.

Poster presentation and co-author, Macaque whole blood samples for flow cytometric analysis of antigen-specific intracellular TNF $\alpha$  and IFN $\gamma$  production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells by Keeney T, Nomura L, Maecker H, Sastry KJ. 19<sup>th</sup> Annual Symposium

on Non-human Primate Models for Aids, San Juan, Puerto Rico, November 7-10, 2001.

**Seminar Invitations from Other Institutions**

N/A

**Lectureships and Visiting Professorships**

N/A

**Other Presentations (state, local) Conferences**

N/A

**PROFESSIONAL MEMBERSHIPS/ACTIVITIES**

**Professional Society Activities, with Offices Held**

**Local/state**

N/A

**National and International**

Member - American Association for Advancement of Science

Member - American Association of Immunologists

Member - International Society for Antiviral Research

**OTHER**

**RESEARCH EXPERIENCE:**

1. Safe handling of HIV infected tissue culture cells, blood and tissue samples from AIDS patients in institutional biohazard level 3 facility using level 3 procedures.
2. Evaluation of nucleotide analogues and peptides from HIV-1 as anti-HIV agents by: Reverse transcriptase assay, Western blot analysis, syncytium formation, MT-4/MTT dye reduction assay and neutralizing antibody analysis.
3. Assay of T- and B-cell responses to HIV peptides in mice, rhesus monkeys, chimpanzees and humans. Determination of T-cell proliferation activity by <sup>3</sup>H-thymidine incorporation assay and cytotoxic T-lymphocyte (CTL) activity by <sup>51</sup>Cr-release assay.
4. Screening of HIV peptides for *in vivo* induction of HMC-restricted, CD8<sup>+</sup> cytotoxic T-lymphocyte responses in mice.
5. PCR analysis of HIV infection in PBMC's, serum, and cerebrospinal fluid from HVI positive individuals.
6. Animal handling: various types of immunization (i.v., i.p., s.c, and i.m. and foot pads), retro-orbital plexus for blood sampling from mice.
7. Analytical techniques include ultracentrifugation, spectrophotometry, scintillation counting, radio-immunoprecipitation analysis, ELISA, immunofluorescence etc.
8. Isolation characterization of DC's from blood and using peptide pulsed-DC's as vaccine.
9. Intracellular Elispot assay for  $\gamma$ -IFN and TNF- $\alpha$ .
10. Designing of new delivery systems for synthetic peptide antigens to induce specific immune responses



## Inhibition of Human Immunodeficiency Virus Type 1 Infection and Syncytium Formation in Human Cells by V3 Loop Synthetic Peptides from gp120

PRAMOD N. NEHETE, RALPH B. ARLINGHAUS, AND K. JAGANNADHA SASTRY\*

*Department of Molecular Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030*

Received 14 May 1993/Accepted 21 July 1993

Because V3 loop-specific antibodies have been shown to inhibit human immunodeficiency virus type 1 (HIV-1) infection of human cells and because specific mutations in the V3 loop render the virus ineffective for infection and syncytium formation, we tested the anti-HIV effects of V3 loop peptides from different HIV-1 strains. We obtained evidence that V3 loop synthetic peptides of 8 to 15 amino acids at nanogram concentrations efficiently blocked HIV-1 IIIB infection of several human T-cell lines and of freshly prepared normal human T cells. More importantly, syncytium formation by three different primary clinical HIV isolates was inhibited by the V3 loop peptide from HIV-1 IIIB at a concentration of 1  $\mu\text{g/ml}$ . Concentrations of V3 peptides up to 50  $\mu\text{g/ml}$  were not toxic to any of the human cells studied. Additionally, V3 peptides incubated in normal human serum or plasma exhibited biological and physical stability for up to 24 h. Taken together, these results suggest that the V3 loop peptides have medical utility as therapeutic reagents to either prevent HIV-1 infection in humans or reduce the spread of virus infection in HIV-infected individuals. These findings are especially significant because a number of reports in the literature indicate that the V3 loop region in gp120 plays an important role in the initial stages of HIV-1 infection of cells.

The envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) plays a central role in the life cycle and pathogenesis of the virus. It is synthesized as a precursor polypeptide, gp160, which is cleaved by a host cell protease to generate the surface envelope glycoprotein gp120 and the transmembrane glycoprotein gp41 (8, 18). Viral infection begins with the envelope glycoprotein binding to the CD4 receptor on uninfected cells. This step is followed by a critical second step in which the lipid bilayer of the virus fuses with the lipid bilayer of the target cell (16, 26). Expression of gp120 at the cell surface has also been shown to induce cell-to-cell fusion, or syncytium formation, in appropriate cell types bearing CD4 (14, 25).

Although the mechanism by which the HIV-1 envelope glycoproteins trigger membrane fusion is poorly understood, it is well established that proteolytic processing of the precursor gp160 is required for HIV-1 infection of cells (8, 27). Several reports in the literature have shown that the V3 loop region of gp120 is essential for HIV-1 infection because (i) specific mutations introduced into the V3 loop will inhibit infectivity and syncytium formation (3, 4, 9), (ii) the V3 loop region is the target for a trypsin-related protease on the host cell surface (6, 11, 20), and (iii) antibodies to the principal neutralizing domain in the V3 loop region inhibit HIV-1 infection of cells without interfering with the binding of HIV-1 gp120 to its cellular receptor, the CD4 molecule (10, 12, 15, 21, 23). It has therefore been proposed that after gp120 present on the viral surface binds to the CD4 receptor on the target cell membrane, the V3 loop region in gp120 is proteolytically cleaved by a cell surface protease, leading to a conformational change in the gp120-gp41 protein complex on the viral surface (6, 11, 20). Such a change is hypothesized to be responsible for exposing the fusogenic domain of the transmembrane protein gp41,

resulting in the fusion of the viral-particle membrane with the cell membrane.

Recently, Koito et al. (11) reported that synthetic peptides (of 24 and 36 amino acids) corresponding to the principal neutralizing domain of HIV-1 IIIB inhibit syncytium formation by interacting with a proteinase-like molecule at the cell surface. However, De Rossi et al. (2) reported that synthetic peptides (of 24 amino acids) from principal neutralizing domains of HIV-1 MN and IIIB enhanced infection of Molt-3 cells by different HIV-1 strains, presumably through a CD4-dependent mechanism. We examined the effects of synthetic peptides from the V3 loop regions of different HIV-1 strains and observed that peptides of 8 to 15 amino acids exhibit efficient inhibition of virus infection of various human T cells as well as of virus-induced syncytium formation.

We tested a 15-amino-acid synthetic peptide, designated R15K, from the V3 loop region in HIV-1 IIIB for its effect on infection of the human T lymphoblastoid cell lines MT-4 and CEM by HIV-1 IIIB. In these experiments, the cells were pretreated with various concentrations of the peptides for 15 min before being infected with the virus. The amount of virus used is equivalent to 10 infectious virus particles per cell. This calculation was based on endpoint dilution analysis of virus stock and is equal to approximately 10 times the dose required for maximum cytopathic effect on MT-4 cells. We measured the infectivity in terms of virus production by assaying for reverse transcriptase activity in the culture medium after 7 days of infection as described by Popovic et al. (22). While infection of MT-4 cells was blocked by 90% at a peptide concentration of 150 ng/ml (0.15  $\mu\text{g/ml}$ ) (Fig. 1A), as little as 8 ng/ml of R15K completely inhibited infection of CEM cells by HIV-1 IIIB (Fig. 1B). The lack of cytotoxicity of R15K peptide at these concentrations was demonstrated in MT-4 cells by determining total viable cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (1, 5) (Fig. 1A) whereas the viability of CEM cells was estimated by trypan blue dye exclusion (unpublished results). Several peptides

\* Corresponding author.

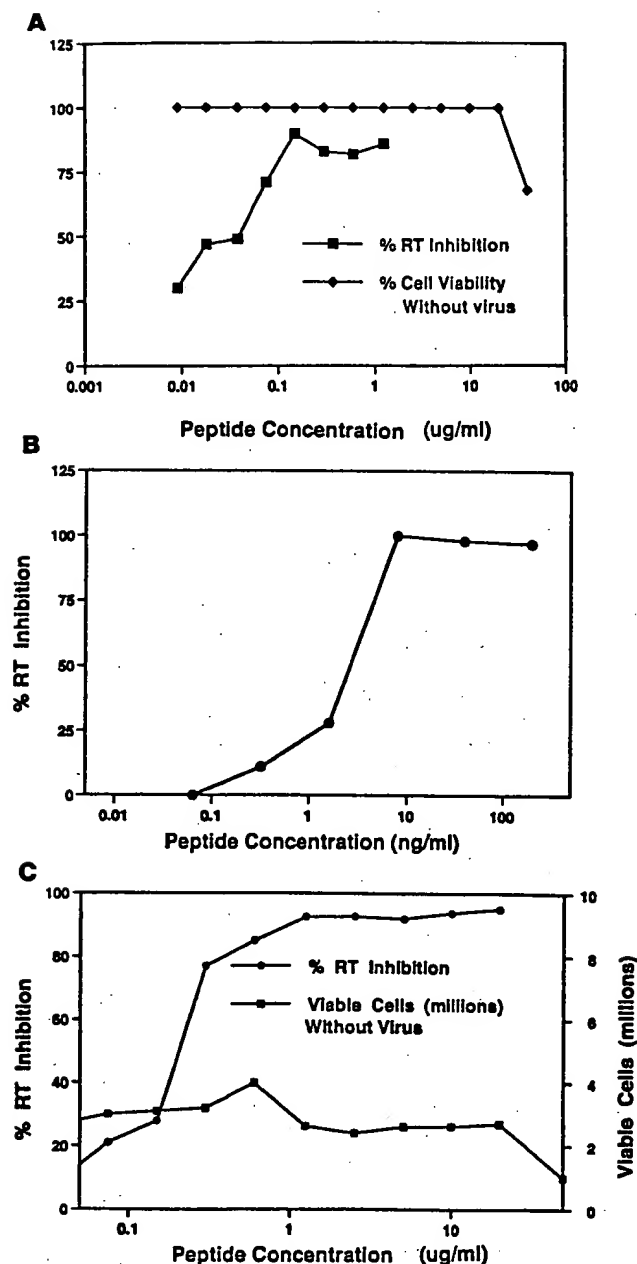


FIG. 1. Peptide R15K inhibits infection of human T-cell lines by HIV-1. (A) MT-4 cells ( $5 \times 10^4$  per well) were preincubated in triplicate wells of a 96-well microtiter plate with various concentrations of the negative control peptide or R15K peptide for 15 min at  $37^\circ\text{C}$  and then infected with HIV-1 at 10 infectious particles per cell. Other controls included cells alone and cells infected with HIV-1 but without R15K. The percent reduction in the amount of reverse transcriptase (RT) activity in the culture medium of cells incubated with various concentrations of R15K was determined 7 days after infection by the method of Popovic et al. (22). The toxicity of R15K for the MT-4 cells was also determined in the same experiment by incubating the uninfected cells with medium alone or with various concentrations of R15K and calculating percent viability by the MTT dye reduction assay (1). (B) CEM cells ( $10^5$  per well) were seeded in a 48-well plate and were infected with HIV-1 at 10 infectious particles per cell in the presence of various concentrations of R15K. The RT activity of the culture medium was measured after 7 days of incubation. (C) Primary human T cells ( $10^5$  per well) were incubated in triplicate wells of a 96-well microtiter plate with medium alone, control peptide S13T, or various concentrations of R15K and then infected with HIV-1 at 10

TABLE 1. Amino acid sequences of synthetic peptides tested for inhibition of HIV infection<sup>a</sup>

Peptide	Source	Amino acid sequence <sup>b</sup>
R15K	HIV IIIB env	<sup>315</sup> RIQRGPGRFVTIGK <sup>329</sup>
N24G	HIV IIIB env	<sup>308</sup> NNTRKSIRIQRGPRFVTIGKIG <sup>331</sup>
R8K	HIV IIIB env	<sup>322</sup> RAFVTIGK <sup>329</sup>
T13Q	HIV RF env	<sup>317</sup> TKGPGRVVIYATGQ <sup>329</sup>
H13N	HIV MN env	<sup>317</sup> HIGPGRFYTTKN <sup>329</sup>
F14F	HIV IIIB env	<sup>215</sup> FEPIPIHYCAFPGF <sup>228</sup>
K14L	HIV IIIB gag	<sup>28</sup> KYKLKHIVWASREL <sup>41</sup>
E12K	HIV IIIB pol	<sup>467</sup> ELELAENREILK <sup>478</sup>
C15P	HIV IIIB pol	<sup>205</sup> CTEMEKEGKISKIGP <sup>219</sup>
S13T	c-mos proto-oncogene	<sup>158</sup> STRTPEDSNSLGT <sup>170</sup>
I15M	Scrambled R15K	IFPGKRTIVAGIRGM

<sup>a</sup> Peptides were synthesized by Merrifield's solid-phase method (19), either on a modified Vega 250 automatic peptide synthesizer or by the bag method as described by Houghten (7).

<sup>b</sup> Amino acid sequences are according to LaRosa et al. (12).

were used as negative control reagents in these experiments (Table 1), including a peptide similar to R15K but with a scrambled amino acid sequence (I15M), one peptide (K14L) from the gag gene, two peptides (E12K and C15P) from the pol gene, one peptide (F14F) from a different region of the env gene, and finally one peptide (S13T) from the c-mos proto-oncogene. No significant level of inhibition of HIV infection was observed with any of these negative control peptides.

We also studied the ability of the R15K peptide at various concentrations to inhibit HIV-1 IIIB infection of primary human T cells. Human T cells isolated from normal donors by standard techniques (17) were stimulated for 72 h with phytohemagglutinin, washed three times, and resuspended at  $10^6/\text{ml}$  in Dulbecco's modified Eagle's medium containing glutamine, 10% heat-inactivated fetal calf serum, and 20 U of human interleukin 2 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml. Aliquots (100  $\mu\text{l}$ ) of the cell suspension were preincubated at  $37^\circ\text{C}$  for 15 min with various concentrations of R15K in triplicate wells of a 96-well microtiter plate. These cells were then infected with 10 infectious particles of HIV-1 IIIB per cell and incubated for 9 days before being assayed for reverse transcriptase activity in the culture medium. R15K inhibited reverse transcriptase production by 50% at 0.12  $\mu\text{g}/\text{ml}$  and by 90% at 1.25  $\mu\text{g}/\text{ml}$  (Fig. 1C). R15K was not toxic to these cells at concentrations up to 20  $\mu\text{g}/\text{ml}$ , but at 50  $\mu\text{g}/\text{ml}$ , 50% of the cells were viable (Fig. 1C). The scrambled R15K peptide (I15M), when tested at different concentrations (0.01 to 1  $\mu\text{g}/\text{ml}$ ) in experiments with MT-4 cells and fresh human T cells, showed no inhibitory effect.

We also tested whether the R15K peptide would directly affect the infectivity of the virus. In these experiments, we preincubated the virus stock with the peptide at 1  $\mu\text{g}/\text{ml}$  for 4 h at  $37^\circ\text{C}$  and subsequently pelleted the virus by ultracentrifugation according to standard protocol. The resulting virus was then compared with untreated virus for infection of MT-4 and H9 cells, and we observed no change in infectivity as measured by both virus-induced cytopathic effect (in MT-4 cells) and reverse transcriptase activity in the culture medium (unpub-

infectious particles per cell. After 9 days, RT activity in culture medium was determined. The total numbers of viable cells at the end of 9 days of incubation in the presence of various concentrations of the peptide were measured by trypan blue dye exclusion.

TABLE 2. Effect of V3 loop peptides from different HIV-1 strains on infection of primary human T cells by HIV-1 IIIB<sup>a</sup>

Cell type and treatment	Reverse transcriptase activity <sup>b</sup> with cells from subject:		
	1	2	3
Uninfected cells	509.52 ± 18	118.75 ± 7	279.44 ± 32
Infected cells plus:			
Medium only	5,425.30 ± 174 (0)	27,142.85 ± 2,116 (0)	36,340.2 ± 1,186 (0)
R15K	407.45 ± 5.2 (100)	5,814.51 ± 212 (79)	6,940.56 ± 214 (81)
N24K	382.25 ± 19 (100)	6,690.26 ± 390 (76)	4,399.40 ± 216 (89)
R8K	1,876.51 ± 44 (72)	7,080.50 ± 215 (74)	8,971.14 ± 602 (76)
T13Q	2,172.19 ± 171 (66)	6,424.20 ± 340 (77)	17,097.25 ± 303 (53)
H13N	1,713.72 ± 44 (75)	6,424.20 ± 340 (77)	19,094.55 ± 183 (48)
S13T	4,948.63 ± 178 (10)	24,824.35 ± 89 (9)	ND

<sup>a</sup> Primary human T cells isolated from fresh human blood were preincubated with peptides (1 µg/ml) and then infected with HIV-1 IIIB as described in the legend to Fig. 1. On day 9, reverse transcriptase activity was measured.

<sup>b</sup> Data are mean counts per minute ± standard error of triplicate samples. The numbers in parentheses are percent inhibition. ND, not determined.

lished results). These results strongly suggest that the infection-inhibitory effects of V3 peptide are not due to an adverse effect on the virus.

Since R15K peptide was effective against infection of normal human T cells, we tested synthetic peptides T13Q and H13N, derived from the V3 loop regions of HIV-1 RF and MN strains, respectively, for their ability to inhibit infection of primary human T cells by HIV-1 IIIB. These results were compared with the inhibitory effects of HIV-1 IIIB V3 loop peptides of various lengths (R15K, N24G, and R8K). All the peptides were added to the cells at a concentration of 1 µg/ml before infection with HIV-1 IIIB. Inhibition of virus infection was determined by assaying the reverse transcriptase activity in culture medium, and the results for cells of three different normal donors are shown in Table 2. The V3 loop peptides from HIV-1 RF and MN strains inhibited HIV-1 IIIB infection by 53 to 77% and 48 to 77%, respectively. In the same experiment, the control peptide S13T did not significantly inhibit infection. Thus, these results indicate that V3 loop

peptides from HIV-1 MN and RF strains exhibit cross-reactivity in terms of infection inhibition. Another important observation from these experiments is that while the 24- and 15-amino-acid peptides (N24G and R15K, respectively) from the V3 loop region of HIV-1 IIIB produced 76 to 100% inhibition, R8K also inhibited HIV-1 IIIB infection of primary human T cells by 72 to 76%. These results clearly indicate that the HIV infection-inhibitory property of the V3 loop peptides from HIV-1 IIIB is within this 8-amino-acid region.

In order to further confirm the HIV infection-inhibitory effect of the 8-amino-acid V3 loop peptide, R8K, we tested infection of four different human T-cell lines by using this peptide at 1 µg/ml. We observed that the R8K peptide was effective in inhibiting HIV-1 IIIB infection of H9 cells by 82%, CEM cells by 85%, MT-4 cells by 88%, and VB cells by 87% as measured by determining the reverse transcriptase activity in the culture medium after 7 days of infection. Additionally, we performed Western blot (immunoblot) analysis to detect p24 protein in these cell lines after infection in the presence or

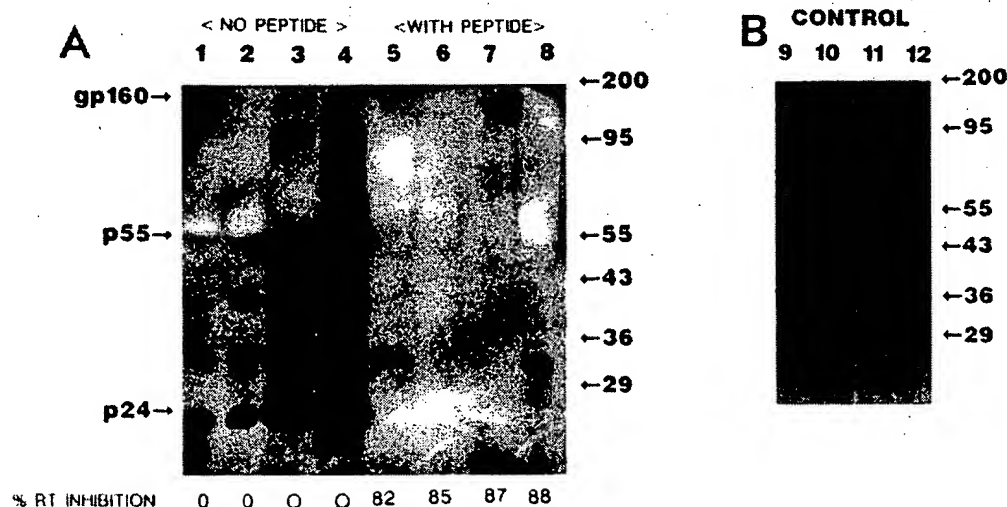


FIG. 2. (A) Western blot analysis of inhibition of expression of HIV-1 gag proteins p55 and p24 by peptide R8K in different human T-cell lines. Total cell lysates of various cell lines infected with HIV-1 IIIB after pretreatment with peptide R8K at 1 µg/ml or no pretreatment were separated on a 10% polyacrylamide gel, transferred to a nitrocellulose filter, and treated with a 1/200 dilution of serum from an HIV-seropositive AIDS patient and <sup>125</sup>I-labeled protein A. Molecular weight markers (in thousands) are shown on the right, and various HIV-1 protein bands are indicated on the left. Samples in lanes 1 to 4 were cell lysates of H9, CEM, VB, and MT-4, respectively, infected with HIV-1 IIIB in the absence of peptide R8K, while lanes 5 to 8 are corresponding cultures infected in the presence of R8K. Percent inhibition of reverse transcriptase (RT) activity by R8K in each of the cell lines is indicated. (B) Western blot analysis of extracts from H9, CEM, VB, and MT-4 cells (lanes 9 to 12, respectively) without virus infection and peptide pretreatment. The experimental method was similar to that described for panel A.

absence of R8K at 1  $\mu\text{g/ml}$ . Nine days postinfection, the cell extracts were prepared and separated on 10% polyacrylamide gels. The proteins transferred onto nitrocellulose filters were reacted with serum from an HIV-positive AIDS patient and  $^{125}\text{I}$ -labeled protein A, and the results are presented in Fig. 2. It is clear from the data that, concurrent with the inhibition of reverse transcriptase in these cell lines, there is also inhibition of p55 and p24 formation. These results, along with the data from Table 2 showing inhibition of infection of human T cells isolated from three different healthy donors, indicate that the effective amino acid sequence is contained within the R8K peptide. Analyses of shorter peptides and of peptides with single-amino-acid changes should provide further understanding of the mechanics of the role of the V3 peptides in the inhibition of HIV-1 infection of human cells.

Since V3 loop peptides from three HIV-1 strains exhibited inhibition of HIV-1 IIIB infection of human T cells (Table 2), we tested their effects on syncytium formation. For these experiments, HeLa-CD4 cells were infected with control vaccinia virus (vSC8) or vaccinia virus recombinants that express gp160 from the IIIB (vPE16) or RF (vRF222) strain of HIV-1 (all reagents were obtained through the AIDS research and reference reagent program of the National Institute of Allergy and Infectious Diseases). HeLa-CD4 cells were plated at a density of  $10^6$  per well of a six-well flat-bottom plate (Costar no. 3506) and allowed to grow for 40 h at  $37^\circ\text{C}$ , at which time the cell monolayer was 80% confluent. Various control or V3 loop peptides at a concentration of 1  $\mu\text{g/ml}$  in 1 ml of medium were added to the monolayers and incubated for 30 min at  $37^\circ\text{C}$ . The peptide solutions were removed, and cell monolayers were washed three times with complete medium. Subsequently, vaccinia virus stocks appropriately diluted to obtain a multiplicity of infection at 100 in a total volume of 1 ml were added, and incubation was continued for an additional 2 h. At this time, the vaccinia virus inoculum was replaced with 3 ml of fresh medium, and cultures were incubated for 18 h. At the end of the incubation period, syncytia were observed under the microscope at a magnification of  $\times 100$  and photographed. While HeLa-CD4 cells preincubated either with medium alone or with peptide before infection with control vaccinia virus showed no syncytia (Fig. 3A and D), cells not preincubated with peptide when infected with vaccinia virus recombinant vPE16 or vRF222 resulted in formation of 80 to 90 syncytia per well of a six-well plate (Fig. 3B and C, respectively). Efficient inhibition of syncytium formation was observed in cells pre-treated with peptides R15K (100% inhibition, no syncytia) and T13Q (80 to 90% inhibition, 8 to 16 syncytia) before infection with recombinant vaccinia viruses vPE16 and vRF222, respectively (Fig. 3E and F). These experiments were repeated at least three times, and each time similar results were obtained with less than 5% difference in numbers of syncytia with various treatments. Thus, V3 loop peptides from HIV-1 IIIB and RF proved effective for inhibition of syncytium formation in cells expressing envelope protein from the respective HIV-1 strain.

We also tested the effectiveness of R15K peptide against syncytium formation by different primary clinical HIV-1 isolates referred to as R006, R042, R043, and R086 (obtained as very early passage of VB cell culture medium from Miles Cloyd, University of Texas Medical Branch, Galveston). HeLa-CD4 cells infected with primary isolates R006, R042, and R043 exhibited 25 to 30 syncytia per well of a six-well plate (Fig. 4A, B, and C), while cells treated with R15K at a concentration of 1  $\mu\text{g/ml}$  showed no syncytium formation (100% inhibition) by these clinical HIV-1 isolates (Fig. 4D, E, and F). However, although syncytia were observed with isolate R086 (28 to 30

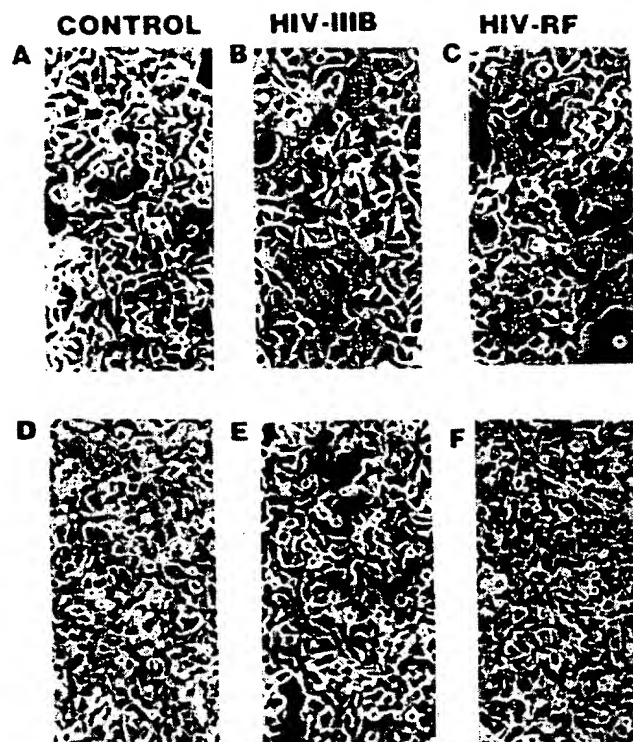


FIG. 3. Inhibition of syncytium formation by V3 loop peptides. Monolayers of HeLa-CD4 cells were incubated with medium alone (A, B, and C), medium containing R15K (D and E), or T13Q (V3 peptide from HIV-1 RF) (F) at 1  $\mu\text{g/ml}$  for 30 min at  $37^\circ\text{C}$ . The cell monolayers were washed before infection with control vaccinia virus (vSC8) (A and D) or recombinant vaccinia virus expressing gp160 from HIV-1 strain IIIB (vPE16) (B and E) or RF (vRF222) (C and F). After 18 h of incubation, cells were observed for syncytia (arrows) under the microscope at  $\times 100$  magnification and photographed. Final magnification,  $\times 90$ .

per well), no inhibitory effect was observed (28 to 30 syncytia per well; unpublished results) with this V3 loop peptide (R15K) at the concentration tested. Thus, the V3 loop peptide R15K was effective not only against laboratory isolates of HIV-1 but also against three of four clinical isolates for inhibiting syncytium formation. These data strongly suggest that the inhibitory effects observed with R15K and other V3 loop peptides are not limited to blocking HIV-1 infection of cells, as R15K can inhibit the fusion of CD4-expressing normal cells with HIV-infected cells expressing the envelope protein, a phenomenon suggested to be responsible for HIV-induced pathology in AIDS patients (13, 14, 25, 27).

These results are in agreement with those of Koito et al. (11), who reported that synthetic peptides (of 24 and 36 amino acids) corresponding to the major HIV-1 IIIB neutralizing epitope of the V3 loop of gp120 (amino acids 303 to 338) inhibited syncytium formation between the HIV-1-infected CCRF-CEM and uninfected Molt-4 cells in a dose-dependent manner. However, in their studies, the 36-amino-acid peptide was effective at a concentration of 100  $\mu\text{M}$  (approximately 300  $\mu\text{g/ml}$ ) while the 24-amino-acid peptide (amino acids 308 to 331) was required at a concentration of 300  $\mu\text{M}$  (approximately 720  $\mu\text{g/ml}$ ) to achieve the same level of inhibition of syncytium formation. However, neither of these peptides was tested by these authors for its capacity to inhibit HIV infection of cells. On the other hand, in our studies, we observed 80 to

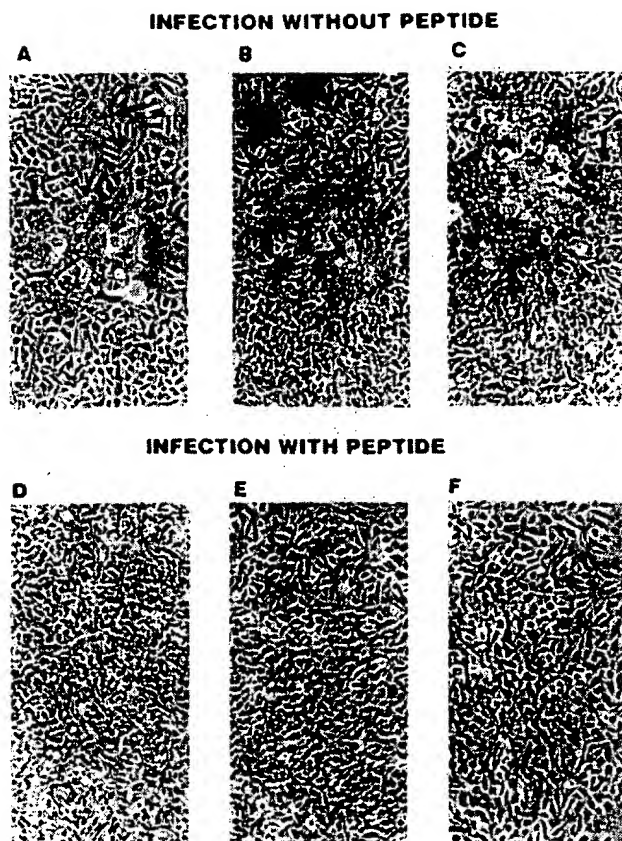


FIG. 4. R15K inhibits syncytium formation by primary clinical HIV-1 isolates. Monolayers of HeLa-CD4 cells were incubated with medium alone (A, B, and C) or medium containing R15K at 1  $\mu$ g/ml (D, E, and F) for 30 min at 37°C. The cell monolayers were washed before infection with primary clinical isolates of HIV-1 R006 (A and D), R042 (B and E), and R043 (C and F). After 72 h of incubation, cells were observed under the microscope for syncytia (arrows) at  $\times 100$  magnification and photographed. Final magnification,  $\times 78$ .

100% inhibition of syncytium formation in HeLa-CD4 cells at much lower concentrations (1  $\mu$ g/ml or less) of V3 loop peptides from two different HIV-1 strains. At this time, we do not know the reason for this discrepancy between our results and those of Koito et al. (11) regarding effective concentrations of V3 peptides for inhibition of syncytium formation. However, in a subsequent publication from the same group it was shown that a 15-amino-acid peptide exhibited strong inhibition of HIV-1 IIIB-induced syncytium formation at only 1  $\mu$ M (6). This peptide was from trypstatin, a protease inhibitor which exhibits 40% sequence homology to the 15-amino-acid V3 loop peptide designated R15K in our studies. These results suggest that the length of the peptide and the additional amino acid sequence are important in determining the effective inhibitory concentration of V3 loop peptides.

However, both the results presented here and those of Koito et al. (11) are different from the results reported by De Rossi et al. (2), who observed that synthetic peptides 24 amino acids in length from V3 loop regions of HIV-1 IIIB and MN strains enhanced infection of Molt-3 cells by different HIV-1 strains through a CD4-dependent mechanism. In their experiments, the V3 loop peptide from HIV-1 MN showed significant enhancement of infection of Molt-3 cells by the homologous virus strain at concentrations ranging between 5 and 20  $\mu$ M (approximately 12 to 48  $\mu$ g/ml). In the same study, a 24-amino-

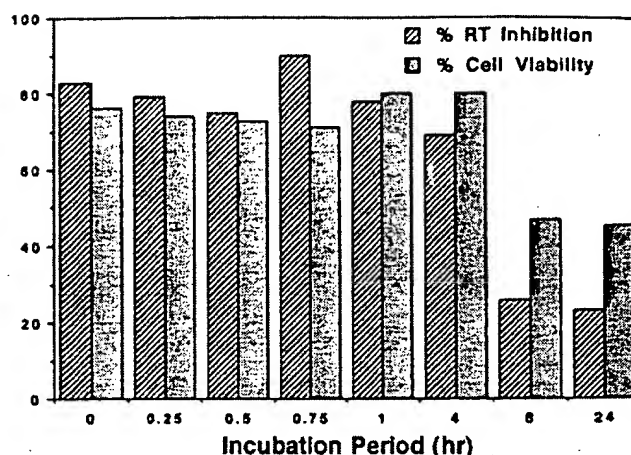


FIG. 5. Biological stability of R15K peptide in fetal calf serum. R15K was incubated at 37°C in fetal calf serum at a concentration of 100  $\mu$ g/ml for 24 h. At different time intervals, aliquots of peptide corresponding to 1  $\mu$ g/ml (final concentration) were added to MT-4 cells in a 96-well microtiter plate. After 15 min of incubation with peptide, the cells were infected with HIV-1 as described in the legend to Fig. 1. Seven days after infection, the total number of viable cells was determined by the MTT dye reduction assay (1), and RT activity in the culture medium was assayed by the method of Popovic et al. (22). The controls included cells incubated in medium alone with and without virus infection and cells infected with virus and incubated with R15K peptide that was not preincubated in fetal calf serum. The results are expressed as percent cell viability or percent reverse transcriptase (RT) activity in MT-4 cells that were infected with HIV-1 in the absence of R15K.

acid peptide from the HIV-1 IIIB strain less efficiently enhanced infection by strain IIIB while a homologous peptide from strain RF was completely ineffective. More importantly, in these studies, a 10-amino-acid peptide corresponding to the middle portion (10 amino acids) of the HIV-1 IIIB V3 loop did not show enhancement of infection. This 10-amino-acid peptide is completely homologous to the C-terminal portion of the R15K peptide in our studies (and the sequence of the R8K peptide also overlaps with this peptide). It is difficult to reconcile our findings with those of De Rossi et al. (2).

In order to gain some understanding of the stability of the inhibitory activity of V3 peptides, we incubated the R15K peptide in fetal calf serum at 37°C. At different intervals during the incubation, aliquots at a final concentration of 1  $\mu$ g/ml (approximately 0.6  $\mu$ M) were tested for inhibition of HIV-1 infection of MT-4 cells. At the end of the 7-day incubation period, both cell viability and the amount of reverse transcriptase present in the culture medium were measured (Fig. 5). It is clear that R15K retained its full-strength inhibitory activity for as long as 4 h of incubation in serum. Even after 24 h of incubation, R15K retained 50% of its inhibitory activity. We obtained similar results with the V3 loop peptide from HIV-1 MN incubated in either fetal calf serum or human serum (unpublished results). The retention of efficient inhibitory effect at these relatively low concentrations bodes well for the use of R15K and other V3 loop peptides as therapeutic reagents. Additionally, we observed that V3 loop peptides incubated in human serum or plasma retained both physical stability (as measured by high-pressure liquid chromatography analysis) and biological activity (unpublished results).

In view of the clinical importance of HIV in AIDS, numerous attempts to develop effective therapeutic reagents have been made in recent years. Most of these approaches were



based on the rationale that virus-specific enzymes such as HIV reverse transcriptase should be suitable targets. Our results provide support for potential therapeutic application of V3 loop peptides because they not only are capable of blocking HIV infection of a number of human cell lines and of primary human T cells isolated from fresh healthy human blood samples but also inhibit cell-to-cell spread of HIV. Also, the V3 loop peptides as a mixture could provide the added advantage of being an immunotherapeutic reagent because, as we recently demonstrated in case of R15K (24), they are capable of inducing HIV-1-specific cytotoxic T-lymphocyte responses that can effectively kill virus-infected cells. Thus, V3 loop peptides have the potential to be medically useful both to block viral spread and HIV-induced pathology and to increase specific cytotoxic T lymphocytes for destruction of virus-infected cells.

This work was supported in part by funds from the National Cancer Institute (AI 29308), the University Cancer Foundation of the University of Texas M. D. Anderson Cancer Center, and PRS Research (4/0020906). All synthetic peptides were prepared in the Synthetic Antigen Core Facility supported by funds from NIH grant CA 16672.

R.B.A. holds the Hubert L. Stringer Chair in Cancer Research. We thank Ron Shonk, the Core Facility peptide chemist, for his assistance. We also thank Miles Cloyd (University of Texas Medical Branch, Galveston) for providing primary HIV-1 isolates. We also thank Linda Jackson for manuscript preparation.

#### REFERENCES

- Baba, M., R. Pauwels, J. Balzarini, J. Arnout, J. Desmyter, and E. D. Clercq. 1988. Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus in vitro. *Proc. Natl. Acad. Sci. USA* 85:6132-6136.
- De Rossi, A., M. Pasti, F. Mammano, M. Panozzo, M. Dettin, C. D. Bello, and L. Chieco-Bianchi. 1991. Synthetic peptides from the principal neutralizing domain of human immunodeficiency virus type 1 (HIV-1) enhance HIV-1 infection through a CD4-dependent mechanism. *Virology* 184:187-196.
- Freed, E. O., D. J. Myers, and R. Risser. 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. *Proc. Natl. Acad. Sci. USA* 87:4650-4654.
- Freed, E. O., D. J. Myers, and R. Risser. 1991. Identification of the principal neutralizing determinant of human immunodeficiency virus type 1 as a fusion domain. *J. Virol.* 65:190-194.
- Harda, S., Y. Koyangi, and Y. Yamamoto. 1985. Infection of HTLV-III/LAV in HTLV-1-carrying cells MT-2 and MT-4 and application in a plaque assay. *Science* 229:563-566.
- Hattori, T., A. Koito, K. Takatsuki, H. Kido, and N. Katunuma. 1989. Involvement of trypsin-related cellular protease(s) in human immunodeficiency virus type 1 infection. *FEBS Lett.* 248:48-52.
- Houghten, R. A. 1985. General method for the rapid solid phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. USA* 82:5131-5135.
- Hu, S. L., S. G. Kosowski, and J. M. Dalrymple. 1986. Expression of AIDS virus envelope gene in recombinant vaccinia viruses. *Nature (London)* 320:537-540.
- Ivanoff, L. A., D. J. Looney, C. McDanal, J. F. Morris, F. Wong-Staal, A. J. Langlois, S. R. Petteway, and T. J. Matthews. 1991. Alteration of HIV-1 infectivity and neutralization by a single amino acid replacement in the V3 loop domain. *AIDS Res. Hum. Retroviruses* 7:595-603.
- Javaherian, K., A. J. Langlois, C. McDanal, K. L. Ross, L. I. Eckler, C. L. Jellis, A. T. Profy, J. R. Rusche, D. P. Bolognesi, S. D. Putney, and T. J. Matthews. 1989. Principal neutralizing domain on the human immunodeficiency virus type 1 envelope protein. *Proc. Natl. Acad. Sci. USA* 86:6768-6772.
- Koito, A., T. Hattori, T. Murakami, S. Matsushita, Y. Maeda, T. Yamamoto, and K. Takatsuki. 1989. A neutralizing epitope of human immunodeficiency virus type 1 has homologous amino acid sequences with the active site of inter-alpha-trypsin inhibitor. *Int. Immunol.* 1:613-618.
- LaRosa, G. J., J. P. Davide, K. Weinhold, J. A. Waterbury, A. T. Profy, J. A. Lewis, A. J. Langlois, G. R. Dreesman, R. N. Boswell, P. Shaddock, L. H. Holley, M. Karplus, D. P. Bolognesi, T. J. Matthews, E. A. Emini, and S. D. Putney. 1990. Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. *Science* 249:932-935.
- Leonard, C. D., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. J. Gregory. 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp 120) expressed in Chinese hamster ovary cells. *J. Biol. Chem.* 265:10373-10382.
- Lifson, J. D., M. B. Reinberg, G. R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K. S. Steimer, and E. G. Engleman. 1986. Induction of CD-4 dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. *Nature (London)* 323:725-728.
- Linsley, P. S., J. A. Ledbetter, E. Kinney-Thomas, and S.-L. Hu. 1988. Effects of anti-gp120 monoclonal antibodies on CD4 receptor binding by the *env* protein of human immunodeficiency virus type 1. *J. Virol.* 62:3695-3702.
- Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* 47:333-348.
- Mannhalter, J. W., W. Borth, and M. M. Eibl. 1986. Modulation of antigen-induced T-cell proliferation by  $\alpha_2$ -M trypsin complexes. *J. Immunol.* 136:2792-2799.
- McCune, J. M., L. B. Rabin, M. B. Feinberg, M. Lieberman, J. C. Kosek, G. R. Reyes, and I. L. Weissman. 1988. Endoproteolytic cleavage of gp 160 is required for the activation of human immunodeficiency virus. *Cell* 53:55-67.
- Merrifield, R. B. 1963. Solid phase peptide synthesis. *J. Am. Chem. Soc.* 85:2149-2154.
- Murakami, T., T. Hattori, and K. Takatsuki. 1991. A principal neutralizing domain of human immunodeficiency virus type-1 interacts with proteinase-like molecule(s) at the surface of Molt-4 clone 8 cells. *Biochim. Biophys. Acta* 1079:279-284.
- Palker, T. J., M. E. Clark, A. J. Langlois, T. J. Matthews, K. J. Weinhold, R. R. Randall, D. P. Bolognesi, and B. F. Hynes. 1988. Type-specific neutralization of the human immunodeficiency virus with antibodies to *env*-encoded peptides. *Proc. Natl. Acad. Sci. USA* 85:1932-1936.
- Popovic, M. M., M. G. Sarngadharan, E. Read, and R. C. Gallo. 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* 224:497-500.
- Rusche, J. R., K. Javaherian, C. McDanal, J. Petro, D. L. Lynn, R. Grimaila, A. Langlois, R. C. Gallo, L. O. Arthur, P. I. Fishchinger, D. P. Bolognesi, S. D. Putney, and T. J. Matthews. 1988. Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. *Proc. Natl. Acad. Sci. USA* 85:3198-3202.
- Sastry, K. J., P. N. Nehete, S. Venkatnarayanan, J. Morkowsky, C. D. Platsoucas, and R. B. Arlinghaus. 1992. Rapid in vivo induction of HIV-specific CD8<sup>+</sup> cytotoxic T lymphocytes by a 15-amino acid unmodified free peptide from the immunodominant V3-loop of gp120. *Virology* 188:502-509.
- Sodroski, J., W. C. Goh, C. Rosen, K. Campbell, and W. A. Haseltine. 1986. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. *Nature (London)* 322:470-474.
- Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman. 1987. PH-independent HIV entry into CD-4 positive T-cells via virus envelope fusion to the plasma membrane. *Cell* 49:659-668.
- Travis, M. M., T. I. Dykers, D. Hewgill, J. Ledbetter, T. T. Tsu, S.-L. Hu, and J. B. Lewis. 1992. Functional roles of the V3 hypervariable regions of HIV-1 gp160 in the processing of gp160 and in the formation of syncytia in CD4<sup>+</sup> cells. *Virology* 186:313-317.

# Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry

J. MICHAEL KILBY<sup>1</sup>, SAM HOPKINS<sup>2</sup>, THOMAS M. VENETTA<sup>2</sup>, BETTY DiMASSIMO<sup>2</sup>,  
GRETCHEN A. CLOUD<sup>3</sup>, JEANNETTE Y. LEE<sup>3</sup>, LESLIE ALLDREDGE<sup>1</sup>, ERIC HUNTER<sup>4</sup>,  
DENNIS LAMBERT<sup>2</sup>, DANI BOLOGNESI<sup>5</sup>, THOMAS MATTHEWS<sup>5</sup>, M. ROSS JOHNSON<sup>2</sup>,  
MARTIN A. NOWAK<sup>6</sup>, GEORGE M. SHAW<sup>7</sup> & MICHAEL S. SAAG<sup>1</sup>

<sup>1</sup>Department of Medicine, University of Alabama at Birmingham, 908 20<sup>th</sup> Street South,  
Birmingham, Alabama 35294-2050, USA

<sup>2</sup>Trimeris, Inc., 4727 University Drive, Durham, North Carolina 27707, USA

<sup>3</sup>Department of Medicine, University of Alabama at Birmingham, 2001 Third Avenue South  
Birmingham, Alabama 35294, USA

<sup>4</sup>Department of Microbiology, University of Alabama at Birmingham, 845 S. 19<sup>th</sup> Street, Room 256,  
Birmingham, Alabama 35294-2170, USA

<sup>5</sup>Department of Surgical Oncology, Duke University Medical Center, Trent Drive,  
Durham, North Carolina 27710, USA

<sup>6</sup>Institute for Advanced Study, Princeton University, Princeton, New Jersey, 08540, USA

<sup>7</sup>Howard Hughes Medical Institute, University of Alabama at Birmingham, 701 S. 19<sup>th</sup> Street, Room 613,  
Birmingham, Alabama 35294, USA

Correspondence should be addressed to J.M.K. and M.S.S.

**T-20, a synthetic peptide corresponding to a region of the transmembrane subunit of the HIV-1 envelope protein, blocks cell fusion and viral entry at concentrations of less than 2 ng/ml *in vitro*. We administered intravenous T-20 (monotherapy) for 14 days to sixteen HIV-infected adults in four dose groups (3, 10, 30 and 100 mg twice daily). There were significant, dose-related declines in plasma HIV RNA in all subjects who received higher dose levels. All four subjects receiving 100 mg twice daily had a decline in plasma HIV RNA to less than 500 copies/ml, by bDNA assay. A sensitive RT-PCR assay (detection threshold 40 copies/ml) demonstrated that, although undetectable levels were not achieved in the 14-day dosing period, there was a 1.96 log<sub>10</sub> median decline in plasma HIV RNA in these subjects. This study provides proof-of-concept that viral entry can be successfully blocked *in vivo*. Short-term administration of T-20 seems safe and provides potent inhibition of HIV replication comparable to anti-retroviral regimens approved at present.**

The therapies now approved for human immunodeficiency virus type 1 (HIV-1) infection inhibit one of two viral-specific enzymes, reverse transcriptase or protease<sup>1</sup>. Although several combination regimens targeting these enzymes have considerable anti-retroviral activity<sup>2-4</sup>, many patients are either intolerant of available agents<sup>5,6</sup> or develop virologic failure because of incomplete viral suppression and the emergence of drug-resistant virus strains<sup>7,8</sup>. Thus, development of new classes of anti-retroviral compounds with different mechanisms of action and toxicity profiles is an important goal.

The HIV-1 envelope glycoprotein consists of two noncovalently associated subunits, a surface glycoprotein (gp120; SU) and a transmembrane glycoprotein (gp41; TM). Portions of gp120 bind to the CD4 receptor as well as to one of the recently characterized chemokine coreceptors on target cells<sup>9</sup>. After the gp120-CD4-coreceptor binding, the gp41 subunit undergoes a conformational change that promotes fusion of viral and cellular membranes, resulting in entry of the viral core into the cell, transport to the nucleus and, ultimately, proviral integration and expression<sup>10</sup>.

Two regions of HIV gp41 contain consensus motifs predic-

tive of hydrophobic alpha-helices<sup>11,12</sup>. These two 'heptad repeat' sequences have been modeled with synthetic peptides, DP107 and DP178, which were found to associate in a 'coiled-coil' secondary structure<sup>13-17</sup>. Recent crystallographic studies of gp41 fragments show that the two heptad repeat domains form a helical bundle containing three members (a trimer) of each domain<sup>10,18,19</sup>. Studies involving these heptad repeat sequences suggest they have a role in the conformational changes essential for membrane fusion of HIV-1 with host cells<sup>20,21</sup>. This process (Fig. 1) has been compared to the 'spring-loaded' mechanism described for influenza virus in which hemagglutinin changes from a loop structure to an extended coiled-coil, moving a 'fusion peptide' into a favorable position for membrane fusion to occur<sup>22,23</sup>. The synthetic peptide mimics (DP107 and DP178) inhibit infection *in vitro* by disrupting the gp41 conformational changes associated with membrane fusion<sup>17,21,24-26</sup>. A 36-amino-acid peptide, corresponding to DP178 and called T-20, was found to be a particularly potent inhibitor of HIV-1 in T-cell lines (50% inhibitory concentration (IC<sub>50</sub>) = 1.7 ng/ml)(ref. 24). This agent was proposed as a promising foremost compound for a new class of antiviral drugs<sup>24</sup>.

**Table 1** Baseline characteristics of subjects

	3 mg (n = 4)	10 mg (n = 5)	30 mg (n = 4)	100 mg (n = 4)	Total (n = 17)
Median age, years (range)	37 (25–47)	37 (30–40)	36 (25–48)	33 (30–36)	36 (25–48)
Gender, male:female	4:0	4:1	4:0	4:0	16:1
Race, Black:White	1:3	2:3	3:1	2:2	8:9
Previously treated: Treatment naive	3:1	5:0	1:3	1:3	10:7
Median days since previous therapy	15 (14–40)	36 (15–210)	30	150	33 (14–210)
Median plasma HIV RNA by bDNA, log <sub>10</sub> (range)	4.86 (4.41–5.19)	5.45 (4.23–5.78)	4.76 (4.25–5.71)	3.83 (3.63–4.82)	4.77 (3.63–5.79)
Median absolute CD4 <sup>+</sup> cells/μl blood (range)	200 (112–350)	279 (105–444)	440 (23–774)	257 (103–658)	212 (23–774)

Preliminary safety and pharmacokinetic studies in animals have indicated that intermittent intravenous T-20 dosing could maintain plasma levels substantially higher than the viral inhibitory concentration (T. Venetta, unpublished data). Here we report the results of the first clinical trial, to our knowledge, examining the safety, pharmacokinetics and anti-retroviral activity of T-20 administered intravenously to HIV-infected humans.

#### Baseline characteristics

We assessed the baseline characteristics of all 17 subjects enrolled in our study (Table 1). Seven patients had not previously received anti-retroviral therapy and ten had received anti-retroviral therapy before the study. One subject (in the 10-mg dose group) dropped out of the study on day 1 (for personal reasons); thus, another subject was added to the 10-mg dose group before subjects were enrolled in the higher dose groups. Four subjects in each of the four dose groups (total,

sixteen subjects) completed the protocol. The median time off of therapy for the ten previously treated patients was 33 days (range, 14–210 days). The median baseline plasma HIV RNA by bDNA assay and CD4 cell counts of the overall study population were 58,884 (4.77 log<sub>10</sub>) copies per ml and 212 cells per ml, respectively.

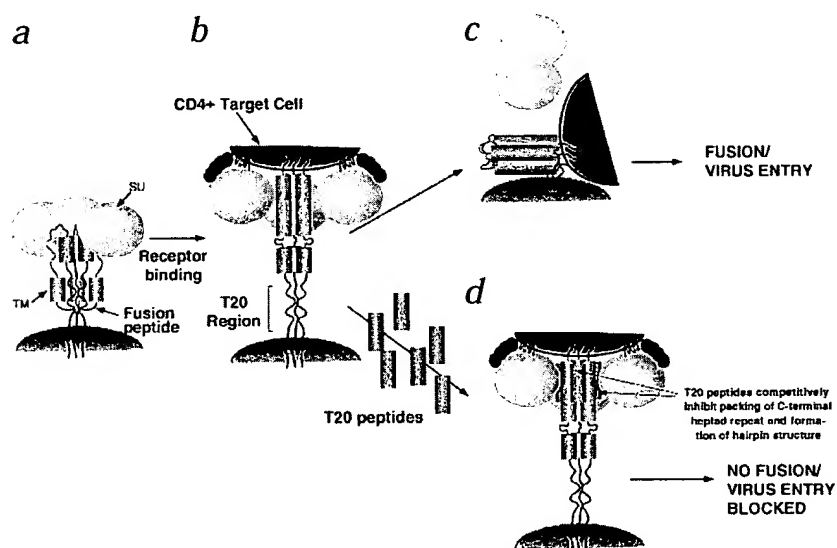
#### Safety and tolerability

Four subjects in each treatment group completed the protocol period as planned except for one subject (10-mg dose group) who left the hospital for one day (day 11) and missed two doses of T-20. No patients were withdrawn from study because of adverse events or toxicity. Four subjects had temperature elevations during the study. The only episodes of fever with

temperatures greater than 38.6 °C could have been caused by other clinical events (community-acquired pharyngitis in one subject and phlebitis at the intravenous catheter site in another). One subject in the 3-mg dose group, one in the 30-mg dose group and two in the 100-mg dose group had isolated episodes of mild-to-moderate headaches; an association with T-20 administration was possible. Transient symptoms noted for which a relationship to T-20 was unclear included mild or moderate pain in the extremities (number of subjects with this symptom (*n* = 3), lymph node tenderness or swelling (*n* = 2), subtle skin discoloration (*n* = 1), dizziness (*n* = 1), and chest pain (*n* = 1). There were no clinically relevant alterations in chemistry or hematologic studies during this trial.

#### Pharmacokinetic measurements

We determined the median pharmacokinetic parameters in each of the dose groups (Table 2). The median *t*<sub>1/2</sub> of T-20 was 1.83



**Fig. 1** Proposed mechanism of T-20 action. **a**, The HIV-1 envelope glycoproteins consist of surface (gp120; SU) and transmembrane (gp41; TM) components. The fusion peptide is in an unexposed position when TM is in the native, non-fusion conformation. **b**, After gp120 binds to a CD4<sup>+</sup> target cell, TM changes conformation, 'unfolding' by a hinge mechanism, and the fusion peptide extends away from the viral surface, inserting into the target cell membrane and forming a 'pre-hairpin intermediate' (ref. 49). **c**, TM resolves into a fusion-active hairpin structure, pulling the cell and viral membranes into close proximity, and allowing fusion and viral entry to occur. **d**, When T-20 is administered, the drug binds to the highly conserved hydrophobic groove of the heptad repeat portions of TM that normally mediate the conformational change from pre-hairpin intermediate to fusion-active hairpin. This prevents membrane apposition, fusion, and virus entry.



**Table 2** Pharmacokinetic measurements: Median (ranges) values after the first (single dose) and seventh (steady-state) intravenous doses of T-20

		3 mg	10 mg	30 mg	100 mg
Maximum observed concentration ( $\mu\text{g/ml}$ )	single-dose*	0.37 (0.31–0.84)	1.61 (1.14–3.10)	5.37 (4.04–7.51)	18.30 (17.39–25.93)
	steady-state*	0.39 (0.31–0.73)	1.53 (1.02–2.77)	5.55 (4.98–10.19)	20.67 (19.12–24.62)
Maximum theoretical concentration ( $\mu\text{g/ml}$ )	single-dose*	0.45 (0.37–0.79)	1.97 (1.24–3.38)	6.25 (4.88–8.04)	21.23 (19.82–31.72)
	steady-state*	0.43 (0.37–0.61)	1.81 (1.16–3.09)	6.82 (5.60–10.82)	24.10 (23.34–28.85)
Area under curve ( $\mu\text{g/hr per ml}$ )	single-dose*	1.19 (0.79–8.73)	4.41 (3.37–9.29)	15.92 (10.83–31.51)	51.70 (48.55–77.05)
	steady-state*	1.11 (0.97–4.62)	4.89 (2.54–10.39)	16.39 (11.87–34.84)	64.43 (48.28–103.12)
Trough concentration ( $\mu\text{g/ml}$ )	single-dose*	0.02 (0.01–0.13)	0.01 (0.004–0.03)	0.04 (0.01–0.07)	0.05 (0.02–0.07)
	steady-state*	0.02 (0.002–0.14)	0.05 (0.02–0.37)	0.32 (0.16–0.70)	1.02 (0.92–2.86)
Half-life of T-20 (hr)	single-dose	1.84 (1.50–7.68)	1.69 (1.54–2.29)	1.77 (1.54–2.72)	1.68 (1.54–1.87)
	steady-state	1.81 (1.75–5.28)	1.91 (1.51–2.33)	1.75 (1.22–2.23)	1.85 (1.43–2.48)
Volume of distribution (liter)	single-dose	6.71 (3.82–8.20)	5.08 (2.97–8.09)	4.81 (3.73–6.14)	4.71 (3.15–5.05)
	steady-state	7.01 (4.96–8.08)	5.66 (3.24–8.60)	4.40 (2.77–5.00)	4.15 (3.46–4.29)

\*,  $P < 0.01$ ; Kruskal-Wallis Test.

hours, which was relatively stable over time and across all dose groups. The median trough concentration of T-20 at steady-state for patients in the 100-mg dose group was 1.02  $\mu\text{g/ml}$ , which is substantially higher than the *in vitro* inhibitory concentration of the drug ( $\text{IC}_{50} = 1.7 \text{ ng/ml}$ ) (ref. 24).

#### Measurements of anti-retroviral activity

There was a significant decline in the level of plasma HIV RNA from day 1 to day 15 of the study when all 16 subjects were considered together (the median change for all dose groups combined was  $-0.39 \log_{10}$ ;  $P < 0.05$ ). We determined the median change in plasma HIV RNA level from baseline for the duration of the study for each treatment group (Fig. 2). There was a significant difference between dose groups with respect to HIV RNA levels over time ( $P < 0.001$ ), indicating a dose-response relationship between the amount of drug administered and the antiviral effect. As predicted from pharmacokinetic studies in animals, the 3-mg and 10-mg dose groups had minimal, if any, changes in HIV RNA levels ( $-0.08 \log_{10}$  and  $-0.05 \log_{10}$ , respectively). The 30-mg dose group had a median decline of  $-0.62 \log_{10}$  over the 14 days of intermittent therapy, and in the 100-mg dose group, all

four patients had a decline in HIV RNA levels below the detection limit of the bDNA assay (500 copies per ml). These samples were re-tested using an ultrasensitive PCR-based HIV RNA assay (Fig. 3). The median change in plasma viral load in these patients was  $-1.96 \log_{10}$  by day 15 of the study, at which time the absolute plasma viral RNA levels were 320, 647, 1197 and 1721 copies per ml for patients A–D, respectively.

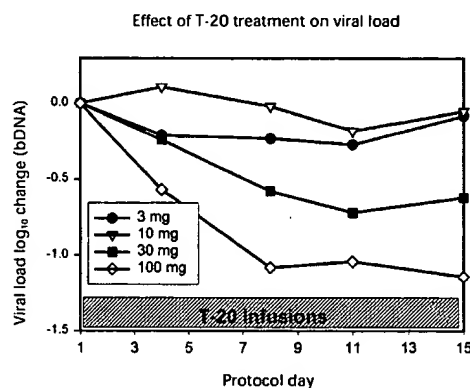
We summarized the changes in plasma HIV RNA and CD4+ lymphocyte counts for all 16 patients who completed the study assessments (Table 3). There was a statistically significant decline in plasma viral RNA between day 1 and day 15 for the 100-mg dose group, whether the bDNA or the ultrasensitive PCR assay was used ( $P < 0.05$  for both assays). Plasma viral load in this dose group rebounded to near-baseline levels (median 3.73  $\log_{10}$  HIV RNA copies per ml, by bDNA assay) a week after T-20 treatment was stopped (day 21). There was a relatively wide variation in CD4 lymphocyte counts in all dose groups between baseline and day 15, ranging from a decrease of 104 cells to an increase of 177 cells. No statistically significant changes in absolute CD4 counts were observed over the 2-week period of study.

#### Viral dynamics

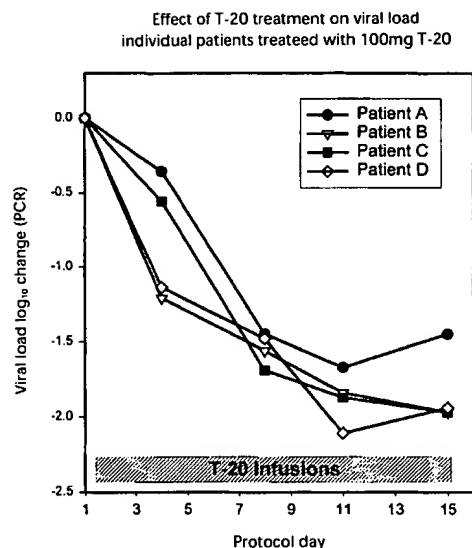
The substantial declines in plasma virus in the 100-mg dose group warranted a kinetic analysis of virus decline, and by inference, an estimation of the relative antiviral efficacy of the T-20 agent compared with other anti-retrovirals. The kinetics of the initial phase of virus elimination was evaluated during the first week of treatment as described<sup>27–29</sup>. There was remarkable uniformity (0.48–0.56) in the viral decay slopes of the four patients treated with the 100 mg dose. These rates correspond to a composite half-life of  $1.4 \pm 0.1$  days for plasma virus and virus-producing cells. The magnitude of virus decline during the first phase of elimination was also quite uniform, amounting to a median of  $-1.52 \log_{10}$  on Day 8. By day 15, the median reduction in virus load was  $-1.96 \log_{10}$  (Fig. 3).

#### Discussion

This study describes the safety, pharmacokinetics, and anti-retroviral activity of T-20, a new synthetic peptide that blocks HIV-1 gp41-mediated membrane fusion. This was a Phase I/IIb, open-label trial involving 16 subjects treated for a 2-week period. There were no significant drug-related side-effects, and



**Fig. 2** Median plasma viral load changes from baseline for subjects in all four dose groups: Filled circle, 3 mg; open triangle, 10 mg; filled square, 30 mg; open diamond, 100 mg. Shaded bar, T-20 treatment period. There was a significant difference from baseline on day 15 for all groups ( $P < 0.05$ ), as well as for the 100-mg dose group considered alone ( $P < 0.05$ ). Viral loads measured by bDNA assay<sup>45,46</sup>.



**Fig. 3** Plasma viral load changes from baseline for the four subjects in the 100-mg dose group ( $P < 0.05$  for difference from baseline on day 15). Filled circle, patient A; open triangle, patient B; filled square, patient C; open diamond, patient D. Shaded bar, T-20 treatment period. Viral loads measured by ultrasensitive PCR assay<sup>47</sup>.

the anti-retroviral activity of the agent was unequivocal and of substantial magnitude ( $-1.96 \log_{10}$  reduction in plasma viral load at the highest dose level). This study thus represents the first clinical demonstration, to our knowledge, that selective inhibition of HIV-1 fusion and entry in humans can lead to biologically significant reductions in plasma virus load. The findings provide proof-of-concept for new therapeutics targeting this essential step in the life-cycle of HIV and other fusogenic, enveloped viruses.

The magnitude and kinetics of virus decline indicate that the efficiency of suppression of *de novo* virus infection by T-20 is comparable to that achieved with anti-retroviral treatments available now<sup>2-4,27-31</sup>. Here, administration of T-20 led to a fall in plasma HIV RNA of about  $2 \log_{10}$  (99%) with a half-life of  $1.4 \pm 0.1$  days. The slope of virus decline is a measure of the anti-retroviral potency of a drug<sup>32,33</sup>; a more potent drug should lead to a faster decline. Monotherapy with protease inhibitors has resulted in viral decay half-lives of  $1.8 \pm 0.9$  days<sup>27</sup>,  $2.1 \pm 0.4$  days<sup>28</sup>, and  $1.6 \pm 0.6$  days<sup>30</sup> for productively infected cells. Triple-drug therapy using two reverse transcriptase inhibitors and one protease inhibitor led to a half-life of  $1.1 \pm 0.4$  days<sup>34</sup>. Even studies involving drug-naïve patients treated with four-drug regimens of highly active anti-retroviral therapy (saquinavir, zidovudine and lamivudine; amprenavir, abacavir, zidovudine and lamivudine) showed slopes of plasma virus decline of 0.43/day and 0.44/day, respectively, corresponding to composite plasma virus

and productive cell half-lives of 1.72–1.80 days (D. Ho, personal communication and abstract S49, 5th Conference on Retroviruses and Opportunistic Infections, Chicago, Illinois, 1998).

Our findings indicate that over a 2-week treatment period, the efficacy of T-20 in blocking *de novo* virus infection is comparable to that of protease and RT inhibitors. After 14 days of therapy, virus was still detectable in the plasma of each patient, even those receiving the highest dose of T-20. This observation is analogous to the results with the highly active combination regimens now available, for which the time necessary to achieve plasma HIV RNA levels less than 25–50 copies per ml is typically longer than 12 weeks<sup>31,34</sup>. The similarity, in kinetics and the magnitude of virus decline, between T-20 and agents now available demonstrates that they are equally accessible to the principal sites of virus replication responsible for sustaining steady state plasma virus loads, which are prognostically very important.

Other strategies have been pursued in an attempt to inhibit viral entry. Sulfated polyanions, such as dextran sulfate or heparin, inhibit HIV infection *in vitro* apparently because of non-specific interference with HIV glycoprotein binding to lymphocytes<sup>35,36</sup>. However, studies of dextran sulfate in humans demonstrated substantial toxicity and resulted in paradoxical increases in virus load<sup>37</sup>. Soluble CD4 and CD4-IgG also effectively inhibit HIV replication *in vitro*<sup>38</sup>, but clinical trials of these agents did not demonstrate consistent anti-retroviral activity<sup>39-41</sup>. These shortcomings may have been due to differences between laboratory-adapted and primary isolates of HIV-1 or the presence of factors in plasma that inhibit the activity of these agents *in vivo*, or they may relate to the binding of gp120 to secondary cellular HIV-1 receptors in addition to the CD4 receptor. Strategies aimed at blocking the interaction of HIV-1 gp120 with the CXCR4 chemokine receptor (which preferentially binds to syncytium-inducing viral isolates associated with advanced or rapidly progressive HIV infection) were promising in preliminary *in vitro* studies<sup>42,43</sup>. Our results with T-20 provide additional support for exploring agents that target membrane fusion and virus entry; future investigations will be further aided by recent detailed characterization of the viral envelope glycoprotein structure and binding characteristics<sup>9</sup>.

The development of a suitable outpatient drug delivery system for T-20 could result in substantial clinical benefit as a result of inhibiting this additional step in the viral life cycle. Despite the promising anti-retroviral activity in this trial, the complexities involved in developing a suitable oral formulation for peptide-based agents may initially limit the widespread clinical application of T-20. Studies with insulin, another par-

**Table 3** Effects of T-20 treatment

Dose group	Viral load		CD4 counts	
	Day 1 viral load (median log <sub>10</sub> ; range)	Median change on day 15 (log <sub>10</sub> ; range)	Entry* CD4 count (median cells/μl; range)	Median change on day 15 (range)
3 mg (bDNA)	4.86 (4.41, 5.19)	-0.08 (-0.40, +0.11)	222 (144, 392)	-27 (-93, +4)
10 mg (bDNA)	5.45 (4.23, 5.78)	-0.05 (-0.21, +0.31)	324 (125, 463)	+24 (-104, +177)
30 mg (bDNA)	4.76 (4.25, 5.71)	-0.62 (-0.77, +0.53)	400 (115, 814)	-11 (-40, +46)
100 mg (bDNA)	3.83 (3.63, 4.82)	-1.14 <sup>b,c</sup> (-2.13, -0.93)	267 (119, 589)	+80 (-13, +109)
100 mg (PCR)	4.74 (4.45, 5.05)	-1.96 <sup>c</sup> (-1.97, -1.45)		

\*Mean of screening and day 1 values <sup>a</sup>All values less than 500 copies/ml on bDNA assay <sup>c</sup>Difference from baseline,  $P < 0.05$

## ARTICLES

enterally administered protein, have demonstrated controlled plasma pharmacokinetics and improved clinical outcomes when the drug is delivered by continuous subcutaneous administration<sup>44</sup>. The pharmacokinetic profile of T-20 (plasma  $t_{1/2}$ , 1.7–2.0 hours) indicates that either intermittent or continuous parenteral administration may be possible. Larger, multicenter, randomized trials are in development. These studies will evaluate the safety, pharmacokinetics and antiviral activity of T-20 when it is given by continuous subcutaneous infusion as a component of a 'rescue regimen' for patients who have had virologic failure while receiving anti-retroviral therapies now available. HIV isolates with reduced susceptibility to T-20, derived from serial *in vitro* viral passage in the presence of the compound, have been characterized<sup>26</sup>. Further clinical studies are underway to assess the potential for selection of variants resistant to T-20 *in vivo* as well as the possibility that anti-T20 antibody responses could alter the efficacy of the compound. Nonetheless, blocking of membrane fusion and viral entry by T-20 establishes a new class of antiviral therapy that exhibits potent activity *in vivo* and is unlikely to be cross-resistant with currently available agents.

### Methods

**Study patients.** Adult HIV-infected subjects were screened at the University of Alabama at Birmingham (UAB) 1917 Clinic, and were eligible for this study if they had at least two viral load determinations of 10,000 or more copies of HIV-1 RNA per ml of plasma as determined with the branched-chain DNA assay (Chiron, Emeryville, California)<sup>45,46</sup>, a CD4 count greater than or equal to 100 cells per mm<sup>3</sup>; and either never received anti-retroviral therapy or had discontinued all anti-retroviral therapy at least 14 days before starting the medication for this study. Patients were excluded if they had a poor clinical status (Karnofsky score less than 80%), clinically unacceptable screening hematologic or blood chemistry results, or signs of an active opportunistic infection or invasive neoplasm. All subjects provided informed consent for participation in the study which was approved by the UAB institutional review board.

**Study design and patient evaluation.** This study was a single-site, open-label, dose-escalation trial of single-agent therapy with intravenous T-20 administered in the General Clinical Research Center (GCRC) in-patient unit at UAB. We planned to study four patients at each dose level (3 mg, 10 mg, 30 mg and 100 mg, all twice daily) and add as many as four more patients at any dose level if significant toxicity or intolerance were encountered. Two baseline viral load determinations and two CD4 counts were obtained during the 30-day screening period. All patients who were receiving anti-retroviral therapy before the trial underwent a 'washout' phase lasting at least 14 days. Each patient received a single intravenous infusion of T-20, at his or her respective dose level, 3 days before beginning the 2-week daily dosing schedule (day -3). This initial test dose was used for preliminary safety and pharmacokinetics assessments. On day 1, patients began intermittent T-20 infusions, for 20 minutes every 12 hours for 14 consecutive days (days 1–14). Vital signs, physical examinations, blood chemistries, and complete blood counts were monitored during the study. Blood for viral load determinations was obtained on days -14, -3, 1 (before T-20 dosing), 4, 8, 11, 15 and 21; CD4<sup>+</sup> lymphocyte counts were determined on days -14, 1, 4, 8 and 15. Patients were discharged from the hospital on day 15 and returned for an outpatient follow-up visit on day 21.

### Virologic measurements

Anti-retroviral activity was first assessed by measuring plasma viral load using the branched-chain DNA method (bDNA; Chiron, Emeryville, California)<sup>45,46</sup>, which has a detection limit of 500 copies of HIV-1 RNA per ml. Because viral RNA levels fell below detectability by the bDNA assay in all four patients who received the highest dose of T-20 (100 mg), plasma samples from these patients were re-tested using a quantitative ultrasensitive RT-PCR HIV RNA assay<sup>47</sup> (Roche, Somerville, New Jersey) with a detection limit of 40 copies per ml.

### Pharmacokinetic studies

Frequent blood sampling was obtained twice, on day -3 and on day 3, for single-dose and steady-state pharmacokinetic studies, respectively. Blood samples on these days were drawn immediately before and 0.5, 1, 2, 4, 6, 8, 12 and 24 hours after the completion of the 20-minute drug infusion. T-20 levels were determined using a sandwich capture ELISA assay (T.M.V., manuscript in preparation).

### Statistical analysis

We used the Wilcoxon rank sum test to compare dose levels with respect to CD4 count and viral load at baseline, and the paired t-test to compare values from day 1 with those from day 15 for CD4 count and viral load within each dose level. We evaluated the effect of T-20 dose groups and time on study on the CD4 count and viral load using 2-way analyses of variance. Pharmacokinetic data were fit to a one-compartment model utilizing SAS non-linear procedures<sup>48</sup>. Area under the curve, half-life, and the volume of distribution were calculated using the estimated coefficients derived from the non-linear regression procedure. All pharmacokinetic measurements, viral load results and CD4 counts are expressed as medians with the corresponding ranges in parentheses; changes in the last two parameters are expressed as median change from baseline.

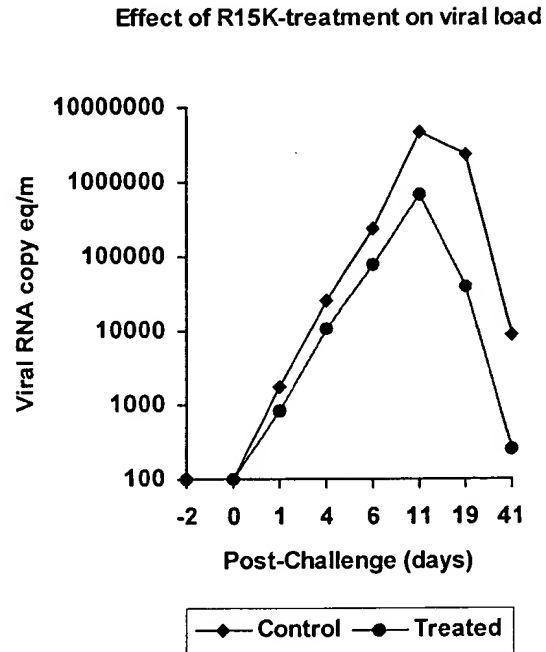
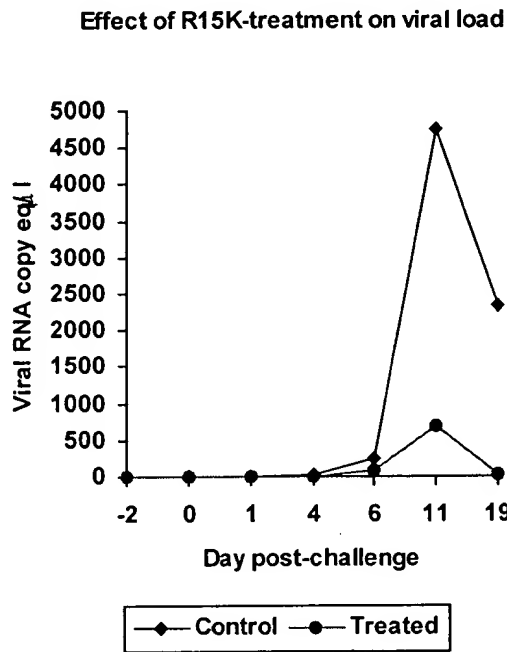
### Acknowledgments

The authors thank the study participants; R.P. Bucy for advice and assistance with figures; and Y. Niu for technical assistance. This study was supported by grants from Trimeris, the UAB Center for AIDS Research (NIH P30 A127767), and the UAB General Clinical Research Center (NCRR MO1 RR00032). E.H., D.B. and T.M. own stock in Trimeris, have served on the Board of Directors and are consultants for the company; M.S.S. is a consultant for the company.

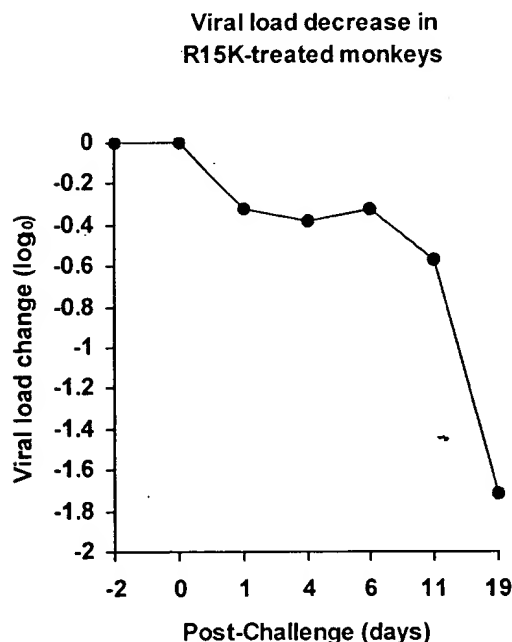
RECEIVED 23 JULY; ACCEPTED 5 OCTOBER 1998

1. Carpenter, C.C. *et al.* Anti-retroviral therapy for HIV infection in 1998. *J. Am. Med. Assoc.* **280**, 78–86 (1998).
2. Eron, J.J. *et al.* Treatment with lamivudine, zidovudine, or both in HIV-positive patients with 200 to 500 CD4<sup>+</sup> cells per cubic millimeter. *N. Engl. J. Med.* **333**, 1662–9 (1995).
3. Markowitz, M. *et al.* A preliminary study of ritonavir, an inhibitor of HIV-1 protease, to treat HIV-1 infection. *N. Engl. J. Med.* **333**, 1534–9 (1995).
4. Hammer, S. *et al.* A randomized, placebo-controlled trial of indinavir in combination with two nucleoside analogs in HIV-infected persons with CD4 cell counts less than or equal to 200 per cubic millimeter. *N. Engl. J. Med.* **337**, 725 (1997).
5. Saag, M.S. Nucleoside analogs: adverse effects. *Hosp. Pract.* **27** (supp), 26–36 (1992).
6. McDonald, C.K. & Kuritzkes, D.R. HIV-1 protease inhibitors. *Arch. Intern. Med.* **157**, 951–959 (1997).
7. Richman, D.D. Resistance of clinical isolates of HIV to anti-retroviral agents. *Antimicrob. Agents Chemother.* **37**, 1207–1213 (1993).
8. Condra, J.H. *et al.* In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* **374**, 569–71 (1995).
9. Kwong, P.D. *et al.* Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648–659 (1998).
10. Chan, D.C., Fass, D., Berger, J.M. & Kim, P.S. Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**, 263–73 (1997).
11. Gallaher, W., Ball, J., Garry, R., Griffin, M. & Montelaro R. A general model of the transmembrane proteins of HIV and other retroviruses. *AIDS Res. Hum. Retroviruses* **5**, 431–40 (1989).
12. Delwart, E., Mosialos, G. & Gilmore, T. Retroviral envelope glycoproteins contain a "leucine zipper"-like repeat. *AIDS Res. Hum. Retroviruses* **6**, 703–6 (1989).
13. Chen, C.H., Matthews, T., McDanal, C., Bolognesi, D. & Greenberg M. A molecular clasp in HIV-1 TM protein determines the anti-HIV activity of gp41 derivatives: implication for viral fusion. *J. Virol.* **69**, 3771–7 (1995).
14. Lawless, M.K. *et al.* HIV-1 membrane fusion mechanism: structural studies of the interactions between biologically-active peptides from gp41. *Biochemistry* **35**, 13697–708 (1996).
15. Matthews, T. *et al.* Structural rearrangements in the transmembrane glycoprotein after receptor binding. *Immunol. Rev.* **140**, 93–104 (1994).
16. Wild, C., Greenwell, T., Shugars, D., Rimskey-Clarke & Matthews T. The inhibitory activity of an HIV-1 peptide correlates with its ability to interact with a leucine zipper structure. *AIDS Res. Hum. Retroviruses* **11**, 323–5 (1995).
17. Wild C., Oas T., McDanal C., Bolognesi D. & Matthews T. A synthetic peptide inhibitor of HIV replication: correlation between solution structure and viral inhibition. *Proc. Natl. Acad. Sci. USA* **89**, 10537–41 (1992).
18. Tan, K., Liu, J.H., Want, J.H., Shen, S., & Lu, M. Atomic structure of a thermostable subdomain of HIV-1 gp41. *Proc. Natl. Acad. Sci. USA* **94**, 12303–308

- (1997).
19. Weissenhorn, W.A., Dessen, A., Harrison, S.C., Skehel, J.J. & Wiley, D.C. Atomic structure of the ectodomain from HIV-1 gp41. *Nature* **387**, 426–30 (1997).
20. Dubay, J., Roberts, S., Brody, B. & Hunter, E. Mutations in the leucine zipper of HIV-1 transmembrane glycoprotein affect fusion and infectivity. *J. Virol.* **66**, 4748–56 (1992).
21. Wild, C. *et al.* Propensity for a leucine zipper-like domain of HIV-1 gp41 to form oligomers correlates with a role in virus-induced fusion rather than assembly of the glycoprotein complex. *Proc. Natl. Acad. Sci. USA* **91**, 12676–80 (1994).
22. Bullough, P.A., Hughson, M., Skehel, J.J. & Wiley, D.C. Structure of influenza haemagglutinin at the pH of membrane fusion. *J. Virol.* **371**, 37–43 (1994).
- Carr, C. & Kim, P.S. A spring-loaded mechanism for the conformational change of influenza haemagglutinin. *Cell* **73**, 823–32 (1993).
24. Wild, C., Greenwell, T. & Matthews, T. A synthetic peptide from HIV-1 gp41 is a potent inhibitor of virus-mediated cell-cell fusion. *AIDS Res. Hum. Retroviruses* **9**, 1051–3 (letter) (1993).
25. Wild, C.T., Shugars, D.C., Greenwell, T.K., McDanal, C.B. & Matthews, T.J. Peptides corresponding to a predictive alpha-helical domain of HIV-1 gp41 are potent inhibitors of virus infection. *Proc. Natl. Acad. Sci. USA* **91**, 9770–4 (1994).
26. Rimsky, L.T., Shugars, D.C. & Matthews, T. Determinants of HIV-1 resistance to gp41-derived inhibitory peptides. *J. Virol.* **72**, 986–992 (1998).
27. Wei, X. *et al.* Viral dynamics in HIV-1 infection. *Nature* **373**, 117–22 (1995).
- Ho, D.D. *et al.* Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**, 123–26 (1995).
29. Herz, A.V.M., Bonhoeffer, S., Anderson, R.M., May, R.M. & Nowak, M.A. Viral dynamics *in vivo*: limitations on estimates of intracellular delay and virus decay. *Proc. Natl. Acad. Sci. USA* **93**, 7247–51 (1996).
30. Perelson, A.S., Neumann, A.U., Markowitz, M., Leonard, J.M. & Ho, D.D. HIV-1 dynamics *in vivo*: virion clearance rate, infected cell life-span, and viral generation time. *Science* **271**, 1582–86 (1996).
31. Montaner, J.S.G. *et al.* A randomized, double-blind trial comparing combinations of zidovudine, didanosine, and zalcitabine for HIV-infected patients. *J. Am. Med. Assoc.* **279**, 930–37 (1998).
32. Nowak, M.A., Bonhoeffer, S., Shaw, G.M. & May, R.M. Anti-viral drug treatment: dynamics of resistance in free virus and infected cell populations. *J. Theor. Biol.* **184**, 203–17 (1997).
33. Bonhoeffer, S., May, R.M., Shaw, G.M. & Nowak, M.A. Virus dynamics and drug treatment. *Proc. Natl. Acad. Sci. USA* **94**, 6971–76 (1997).
34. Perelson, A.S. *et al.* Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature* **387**, 188–191 (1997).
35. Baba, M. *et al.* Mechanism of inhibitory effect of dextran sulfate and heparin on replication of HIV *in vitro*. *Proc. Natl. Acad. Sci. USA* **85**, 6132–36 (1988).
36. Mitsuya, H. *et al.* Dextran sulfate suppression of viruses in the HIV family: inhibition of virion binding to CD4+ cells. *Science* **240**, 646–48 (1988).
37. Flexner, C. *et al.* Pharmacokinetics, toxicity, and activity of intravenous dextran sulfate in HIV infection. *Antimicrob. Agents Chemother.* **35**, 2544–50 (1991).
38. Fisher, R.A. *et al.* HIV infection is blocked *in vitro* by recombinant soluble CD4. *Nature* **331**, 76–78 (1988).
39. Schooley, R.T. *et al.* Recombinant soluble CD4 therapy in patients with AIDS and AIDS-related complex. *Ann. Intern. Med.* **112**, 247–53 (1990).
40. Meng, T.C. *et al.* Combination therapy with recombinant human soluble CD4-immunoglobulin G and zidovudine in patients with HIV infection: a phase I study. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **8**, 152–160 (1995).
41. Shacker, T. *et al.* Phase I study of high-dose, intravenous rCD4 in subjects with advanced HIV-1 infection. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **9**, 145–152 (1995).
42. Chen, J.D., Bai, X., Yang, A.G., Cong, Y. & Chen, S.Y. Inactivation of HIV-1 chemokine co-receptor CXCR-4 by a novel intrakine strategy. *Nature Med.* **3**, 1110–1116 (1997).
43. Donzella, G.A. *et al.* AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. *Nature Med.* **4**, 72–77 (1998).
44. Zinman, B., Tildesley, H., Chiasson, J.L., Tsui, E. & Strack, T. Insulin Lispro in CSII: results of a double-blind crossover study. *Diabetes* **46**, 44043 (1997).
45. Pachl, C. *et al.* Rapid and precise quantification of HIV-1 RNA in plasma using a branched DNA signal amplification assay. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **8**, 446–54 (1995).
46. Cao, Y. *et al.* Clinical evaluation of branched DNA signal amplification for quantifying HIV type 1 in human plasma. *AIDS Res. Hum. Retroviruses* **11**, 353–61 (1995).
47. Schockmel, G.A., Yerly, S. & Perrin, L. Detection of low HIV-1 levels in plasma. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **14**, 179–83 (1997).
48. SAS/STAT User's Guide Version 6, 4th edn., Vol. 1 & 2 (SAS Institute, Cary, North Carolina, 1989).
49. Chan, D. C. and Kim P. S. HIV Entry and Its Inhibition. *Cell* **93**, 681–84 (1998).



**Fig. 1:** The average viral load values (measured by real-time RT-PCR, and shown as copy eq/ml in the panel on left and as copy eq/ml in log scale in the panel on right) for two control and two R15K-treated monkeys. The R15K-treatment (60mg/day/monkey, iv) was initiated on day -2 and continued through day 12 (for a total of 15 days). The control monkeys received sterile saline for the same duration. The monkeys were infected on day 0 with 1000TCID<sub>50</sub> of SHIV<sub>ku2</sub> in 1ml sterile saline.



**Fig. 2:** The average-change in viral load (shown after log-conversion) between control and treated monkeys. The R15K-treatment (60mg/day/monkey, iv) was initiated on day -2 and continued through day 12 (for a total of 15 days). The control monkeys received sterile saline for the same duration. The monkeys were infected on day 0 with 1000TCID<sub>50</sub> of SHIV<sub>ku2</sub> in 1ml sterile saline.

## Reduction of Simian-Human Immunodeficiency Virus 89.6P Viremia in Rhesus Monkeys by Recombinant Modified Vaccinia Virus Ankara Vaccination

DAN H. BAROUCH,<sup>1\*</sup> SAMPA SANTRA,<sup>1</sup> MARCELO J. KURODA,<sup>1</sup> JÖRN E. SCHMITZ,<sup>1</sup> RONALD PLISHKA,<sup>2</sup> ALICIA BUCKLER-WHITE,<sup>2</sup> ALICIA E. GAITAN,<sup>3</sup> REBEKAH ZIN,<sup>3</sup> JAE-HWAN NAM,<sup>4</sup> LINDA S. WYATT,<sup>4</sup> MICHELLE A. LIFTON,<sup>1</sup> CHRISTINE E. NICKERSON,<sup>1</sup> BERNARD MOSS,<sup>4</sup> DAVID C. MONTEFIORI,<sup>3</sup> VANESSA M. HIRSCH,<sup>2</sup> AND NORMAN L. LETVIN<sup>1</sup>

*Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215<sup>1</sup>;*  
*Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Rockville, Maryland 20852<sup>2</sup>;*  
*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892<sup>4</sup>;*  
*and Duke University Medical Center, Durham, North Carolina 27710<sup>3</sup>*

Received 8 December 2000 /Accepted 3 March 2001

Since cytotoxic T lymphocytes (CTLs) are critical for controlling human immunodeficiency virus type 1 (HIV-1) replication in infected individuals, candidate HIV-1 vaccines should elicit virus-specific CTL responses. In this report, we study the immune responses elicited in rhesus monkeys by a recombinant poxvirus vaccine and the degree of protection afforded against a pathogenic simian-human immunodeficiency virus SHIV-89.6P challenge. Immunization with recombinant modified vaccinia virus Ankara (MVA) vectors expressing SIVmac239 *gag-pol* and HIV-1 89.6 *env* elicited potent Gag-specific CTL responses but no detectable SHIV-specific neutralizing antibody (NAb) responses. Following intravenous SHIV-89.6P challenge, sham-vaccinated monkeys developed low-frequency CTL responses, low-titer NAb responses, rapid loss of CD4<sup>+</sup> T lymphocytes, high-setpoint viral RNA levels, and significant clinical disease progression and death in half of the animals by day 168 postchallenge. In contrast, the recombinant MVA-vaccinated monkeys demonstrated high-frequency secondary CTL responses, high-titer secondary SHIV-89.6-specific NAb responses, rapid emergence of SHIV-89.6P-specific NAb responses, partial preservation of CD4<sup>+</sup> T lymphocytes, reduced setpoint viral RNA levels, and no evidence of clinical disease or mortality by day 168 postchallenge. There was a statistically significant correlation between levels of vaccine-elicited CTL responses prior to challenge and the control of viremia following challenge. These results demonstrate that immune responses elicited by live recombinant vectors, although unable to provide sterilizing immunity, can control viremia and prevent disease progression following a highly pathogenic AIDS virus challenge.

A safe and effective human immunodeficiency virus type 1 (HIV-1) vaccine is urgently needed to control the worldwide HIV-1 epidemic. A number of recent studies have demonstrated the importance of virus-specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) in controlling HIV-1 replication in humans and simian immunodeficiency virus (SIV) replication in rhesus monkeys (18, 26, 27, 36). It is therefore widely believed that HIV-1 vaccine candidates should elicit potent virus-specific CTL responses in addition to neutralizing antibody (NAb) responses.

Live, attenuated virus vaccines have been shown to generate CTL and NAb responses capable of controlling a number of pathogenic viral challenges (10, 40, 41). However, significant safety concerns regarding this approach remain. Live, attenuated SIV vaccines have been shown to induce AIDS in neonatal and adult macaques (4, 5). More importantly, humans infected with *nef*-deleted HIV-1 have been reported to develop immunodeficiency and clinical disease (11, 14, 22).

Other vaccine strategies capable of eliciting virus-specific

CTL responses are therefore being evaluated. Approaches that have generated considerable interest include plasmid DNA and recombinant live vectors. We have recently reported that plasmid DNA vaccination elicited high-frequency CTL responses that reduced setpoint viremia following an SIVsmE660 challenge in rhesus monkeys (12). We have also demonstrated that cytokine-augmented DNA vaccination elicited potent immune responses that effectively controlled viremia and prevented clinical disease progression following a pathogenic simian-human immunodeficiency virus SHIV-89.6P challenge (6, 7).

It remains to be determined whether other vaccination modalities, in particular live recombinant vectors, will provide a similar level of protection in monkeys challenged with the highly pathogenic virus SHIV-89.6P (32–34). A number of recombinant live poxviruses have been evaluated for their utility as HIV-1 vaccine candidates. Safety concerns regarding vaccinia virus (31) have led to the development of a number of attenuated poxviruses as vaccine vectors, including NYVAC, fowlpox, canarypox, and modified vaccinia virus Ankara (MVA) (8, 16, 28, 29, 37, 38). MVA is an attenuated form of vaccinia virus that has undergone 570 passages in primary chicken embryo fibroblasts and has genomic deletions that reduce its pathogenicity (23). We have recently reported that a recombinant MVA/*gag-pol* vaccine elicits SIV-specific CTL responses in rhesus monkeys (37). Following a pathogenic

\* Corresponding author. Mailing address: Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Research East Room 113, 330 Brookline Ave., Boston, MA 02215. Phone: (617) 667-2042. Fax: (617) 667-8210. E-mail: dan\_barouch@hotmail.com.

SIVsmE660 challenge, secondary CTL responses were detected associated with a partial control of viremia (38). In another study, vaccination with recombinant MVA/*env*, MVA/*gag-pol*, or MVA/*gag-pol-env* constructs reduced plasma viremia and increased survival following an SIVsmE660 challenge (28, 29).

In the present study, we investigate the ability of recombinant MVA vectors expressing SIV *gag-pol* and HIV-1 *env* derived from the primary patient isolate 89.6 to elicit CTL and NAb responses in rhesus monkeys. We also assess the protection afforded by these immune responses against a highly pathogenic SHIV-89.6P challenge.

#### MATERIALS AND METHODS

**Construction of recombinant MVA vectors.** Open reading frames of SIVmac239 *gag-pol* and HIV-1 89.6 *env* truncated at amino acid 738 were inserted adjacent to the modified H5 promoter in the previously described plasmid transfer vectors pLW-9 and pLW-17, respectively (42, 43). Recombinant MVA/*gag-pol* and MVA/*env* vectors were each produced by homologous recombination, identified by immunostaining of live, infected cell foci, and clonally isolated. The purity of each recombinant virus was assessed by PCR and immunostaining. Expression of the recombinant proteins was determined by radioimmunoprecipitation. The production of Gag particles and surface expression and fusion competence of the expressed Env proteins were demonstrated.

**Vaccination and challenge of rhesus monkeys.** Eight Mamu-A\*01-positive rhesus monkeys were selected for inclusion in this study (20). The monkeys were immunized intramuscularly with  $10^8$  PFU of either control nonrecombinant MVA ( $n = 4$ ) or recombinant MVA vectors expressing SIV *gag-pol* and HIV-1 89.6 *env* at weeks 0, 4, and 21. The monkeys were challenged at week 27 with a 1:500 dilution (estimated 100 50% monkey infective doses [MID<sub>50</sub>]) of the uncloned cell-free SHIV-89.6P stock (33, 34) by the intravenous (i.v.) route. Monkeys were maintained in accordance with National Institutes of Health and Harvard Medical School guidelines.

**Tetramer staining.** Tetramer staining was performed with freshly isolated peripheral blood mononuclear cells (PBMC) from EDTA-anticoagulated whole blood specimens as described (3, 21). Briefly, soluble tetrameric Mamu-A\*01 complexes folded around the SIV Gag p11C epitope (CTPYDINQM) (1, 24) were prepared. One microgram of phycoerythrin-labeled tetrameric Mamu-A\*01/p11C complexes was used in conjunction with fluorescein isothiocyanate-labeled anti-human CD8 $\alpha$  (Leu2a; Becton Dickinson), phycoerythrin-Texas Red (ECD)-labeled anti-human CD8 $\alpha\beta$  (2ST8-5H7; Beckman Coulter), and allophycocyanin-labeled anti-rhesus monkey CD3 (FN18) monoclonal antibodies to stain p11C-specific CD8 $^+$  T cells. A total of 100  $\mu$ l of whole blood from the vaccinated or control monkeys was directly stained with these reagents, lysed, washed, and fixed. Samples were analyzed by four-color flow cytometry with a Becton Dickinson FACS Calibur system, and gated CD3 $^+$  CD8 $\alpha\beta$  $^+$  T cells were examined for staining with tetrameric Mamu-A\*01/p11C complexes.

**CTL assays.** Functional chromium release cytotoxicity assays were performed as described (6). Briefly,  $5 \times 10^6$  washed PBMC from rhesus monkeys were cultured in the presence of 10  $\mu$ g of p11C peptide (CTPYDINQM)/ml (1, 24). On day 3 of culture, 20 U of human recombinant interleukin 2 (Hoffmann-La Roche)/ml was added. On day 12 of culture, peptide-stimulated PBMC were centrifuged over Ficoll (Ficoll-Paque) and assessed as effectors in standard 4-h  $^{51}\text{Cr}$ -release assays containing  $10^4$  target cells/well. Autologous B-lymphoblastoid cell lines pulsed with 1  $\mu$ g of p11C peptide or p11B control peptide (ALSEGCTPYDIN)/ml and labeled overnight with  $^{51}\text{Cr}$  (100  $\mu\text{Ci}/\text{ml}$ ) were used as targets. To measure spontaneous release of  $^{51}\text{Cr}$ , target cells were incubated with 100  $\mu$ l of medium, and for maximum release target cells were incubated with 100  $\mu$ l of 2% Triton X-100. Percent lysis was calculated as follows: [(experimental release - spontaneous release)/(maximum release - spontaneous release)]  $\times$  100.

**Neutralizing antibody assays.** Determination of antibody titers capable of neutralizing SHIV-89.6 and SHIV-89.6P was performed as described (9). Briefly, reduction of virus-induced cytopathic killing of MT-2 cells was measured by Finter's neutral red that is taken up by viable cells. A total of 50  $\mu$ l of cell-free virus containing 500 50% tissue culture infective doses grown in human PBMC was added to multiple dilutions of test plasma in 100  $\mu$ l of growth media in triplicate. These mixtures were incubated for 1 h before the addition of  $5 \times 10^4$  MT-2 cells. Infection led to extensive syncytium formation and virus-induced cell killing in 4 to 6 days in the absence of neutralizing antibodies. Neutralization

titers were calculated as the reciprocal dilution of plasma required to protect 50% of cells from virus-induced killing.

**CD4 $^+$  T-lymphocyte counts and viral RNA levels.** CD4 $^+$  T-lymphocyte counts were determined by multiplying the total lymphocyte count by the percentage of CD3 $^+$  CD4 $^+$  T cells assessed by flow cytometry. Plasma viral RNA levels were measured by a real-time reverse transcriptase PCR amplification assay with a detection limit of 500 copies/ml as described (17) using *gag* primers and probes (39).

**Statistical analyses.** Statistical analyses were performed with GraphPad Prism version 2.01 (GraphPad Software, Inc.). CD4 $^+$  T-lymphocyte counts and viral RNA levels were compared between groups by two-sided Wilcoxon rank sum tests. Day 70 setpoint values were chosen in order to analyze a complete data set prior to the death of any animals. Correlation of prechallenge vaccine-elicited CTLs and day 70 postchallenge setpoint viral RNA levels was assessed by a two-sided Spearman rank correlation test. In all cases, a  $P$  value of  $<0.05$  was considered significant.

#### RESULTS

**Vaccine trial design.** Eight rhesus monkeys (*M. mulatta*) expressing the major histocompatibility complex class I allele Mamu-A\*01 were selected for inclusion in this study (20). These animals were immunized with the control nonrecombinant MVA ( $n = 4$ ) or recombinant MVA vaccines expressing SIV *gag-pol* and HIV-1 *env* derived from the primary patient R5/X4 dual-tropic isolate 89.6 ( $n = 4$ ). Animals received  $10^8$  PFU intramuscularly of control or recombinant MVA vectors at weeks 0, 4, and 21. At week 27, all eight animals were challenged i.v. with SHIV-89.6P. This highly pathogenic virus was derived by in vivo passage of the nonpathogenic virus SHIV-89.6 and has been shown to cause rapid CD4 $^+$  T-lymphocyte loss and clinical AIDS in the majority of naïve rhesus monkeys (7, 32-34).

**Vaccine-elicited immune responses.** Staining CD8 $^+$  T cells with tetrameric MHC class I-peptide complexes followed by analysis by flow cytometry has proven to be an accurate method for quantitating epitope-specific CTLs in freshly isolated whole-blood specimens without the need for in vitro lymphocyte stimulation (3, 21). CTL responses specific for the Mamu-A\*01-restricted immunodominant SIV Gag p11C epitope (CTPYDINQM) (1, 24) were measured by both tetramer staining and functional chromium release cytotoxicity assays. As shown in Fig. 1, p11C-specific CTLs were detected by tetramer staining in all vaccinated animals after the initial immunization. Higher levels were observed 1 week after the week 4 and week 21 boost immunizations, reaching a maximum of 0.2 to 0.8% of circulating CD3 $^+$  CD8 $^+$  T cells. Levels of CTLs following the second and third immunizations were comparable, consistent with the findings in our previous study of CTL responses elicited by recombinant MVA vectors in rhesus monkeys (37, 38). Following each boost immunization, there was a rapid expansion of p11C-specific CTLs followed by a rapid decline to steady-state plateau levels of 0.1 to 0.3% of circulating CD8 $^+$  T cells that persisted over time. Tetramer staining specific for the subdominant HIV-1 Env p41A epitope (YAPPISGQI) (13) was only detected in one animal (H507), and no tetramer staining specific for p11C or p41A was observed in the monkeys that received the control MVA (data not shown). As shown in Table 1, functional chromium release cytotoxicity assays confirmed these tetramer staining data. No NAb responses specific for SHIV-89.6 or SHIV-89.6P ( $<1:20$  titer) were detectable in the control or vaccinated animals at peak immunity or prior to challenge (data not shown).

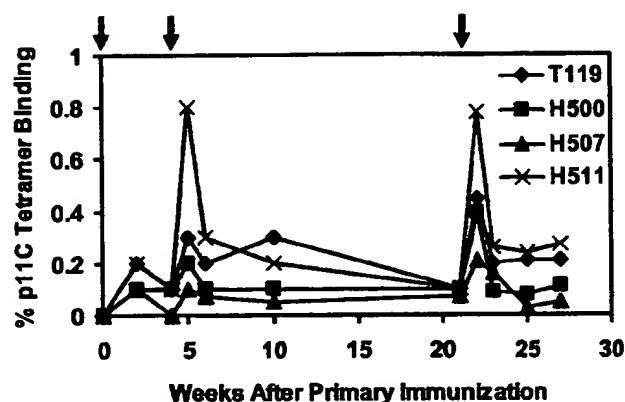


FIG. 1. Vaccine-elicited CTL responses. *Mamu-A\*01*-positive monkeys were immunized at weeks 0, 4, and 21 with recombinant MVA constructs expressing SIV *gag-pol* and HIV-1 89.6 *env*. Vaccine-elicited CD8<sup>+</sup> T-cell responses specific for the immunodominant SIV Gag p11C (CTPYDINQM) epitope (1, 24) were measured by tetramer staining of freshly isolated PBMC (3, 21). The percent CD3<sup>+</sup> CD8<sup>+</sup> T cells that bound the Mamu-A\*01/p11C tetramer is shown. Arrows indicate times of immunization.

**Immune responses following challenge.** Six weeks after the final boost immunization, all eight animals were challenged i.v. with 100 MID<sub>50</sub> of cell-free SHIV-89.6P. All animals were infected by this highly pathogenic viral challenge. As shown in Fig. 2, the control monkeys developed primary p11C-specific CTL responses, reaching a maximum of 0.2 to 2% of circulating CD8<sup>+</sup> T cells on day 14 after challenge. In contrast, the vaccinated monkeys developed higher secondary p11C-specific CTL responses, reaching a maximum of 7 to 20% of circulating CD8<sup>+</sup> T cells on day 14 after challenge. As shown in Table 2, the results of functional chromium release cytotoxicity assays confirmed these tetramer staining data.

NAb responses specific for both SHIV-89.6 and SHIV-89.6P were assessed in MT-2 cell-killing assays (9). As shown in Fig. 3A, no SHIV-89.6-specific NAbs were detected in the plasma

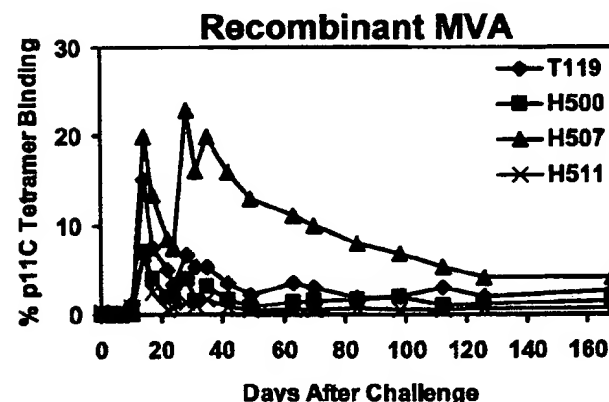
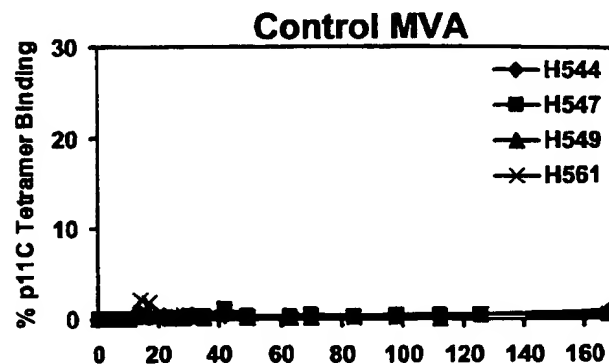


FIG. 2. Secondary CTL responses following challenge. Monkeys were challenged with SHIV-89.6P by the i.v. route on day 0. CD8<sup>+</sup> T-cell responses specific for the SIV Gag p11C epitope were determined by tetramer staining of freshly isolated PBMC at multiple time points (3, 21). The percent CD3<sup>+</sup> CD8<sup>+</sup> T cells that bound the Mamu-A\*01/p11C tetramer is shown.

TABLE 1. Vaccine-elicited CTL responses<sup>a</sup>

Vaccination and monkey	Tetramer binding		Functional cytotoxicity (stimulated PBMC)
	Fresh PBMC	Stimulated PBMC	
Control MVA			
H544	0.0	0	0
H547	0.0	0	1
H549	0.0	0	1
H561	0.0	0	0
Recombinant MVA			
T119	0.5	17	37
H500	0.4	4	26
H507	0.2	39	61
H511	0.8	5	30

<sup>a</sup> SIV Gag p11C-specific CD8<sup>+</sup> T-cell responses as measured by tetramer staining of freshly isolated and peptide-stimulated PBMC and chromium release functional cytotoxicity assays at week 22, which is 1 week following the third immunization. The percent CD3<sup>+</sup> CD8<sup>+</sup> cells that bind tetramer is shown for the tetramer assays. The percent specific lysis at a 5:1 effector-to-target ratio is shown for the cytotoxicity assays.

of the control animals, except for a low titer in monkey H547 on day 70 after challenge. In contrast, SHIV-89.6-specific NAbs were detected in the plasma of two vaccinated animals on day 14 after challenge, and high-titer NAbs (1,350 to 10,804) were observed in the plasma of all four vaccinated animals on day 21 following challenge. This rapid evolution of high-titer NAbs is consistent with a secondary SHIV-89.6-specific NAb response that was primed by the vaccine.

Since NAbs generated by SHIV-89.6 infection exhibit poor cross-neutralizing activity against SHIV-89.6P (9, 25), the vaccine expressing HIV-1 Env 89.6 would not be expected to prime for SHIV-89.6P-specific NAbs. As shown in Fig. 3B, only two control monkeys (H544 and H547) developed SHIV-89.6P-specific NAbs by day 42 after challenge. Surprisingly, all four vaccinated monkeys developed SHIV-89.6P-specific NAbs between days 21 and 42 after challenge. The six animals that developed detectable SHIV-89.6P-specific NAbs had similar peak titers.

CD4 counts, viral RNA levels, and clinical disease progression. As shown in Fig. 4, the control monkeys developed a rapid and profound loss of CD4<sup>+</sup> T lymphocytes between days 7 and 21 after challenge. Monkeys H549 and H561 demonstrated a complete loss of their CD4<sup>+</sup> T lymphocytes, whereas H544 and H547 had significant but incomplete losses of their



TABLE 2. CTL responses following challenge<sup>a</sup>

Vaccination and monkey	Tetramer binding		Functional cytotoxicity (stimulated PBMC)
	Fresh PBMC	Stimulated PBMC	
Control MVA			
H544	0.1	2	2
H547	0.1	1	0
H549	0.7	19	22
H561	1.7	8	11
Recombinant MVA			
T119	7.5	48	29
H500	3.9	49	37
H507	13.5	59	34
H511	2.5	42	28

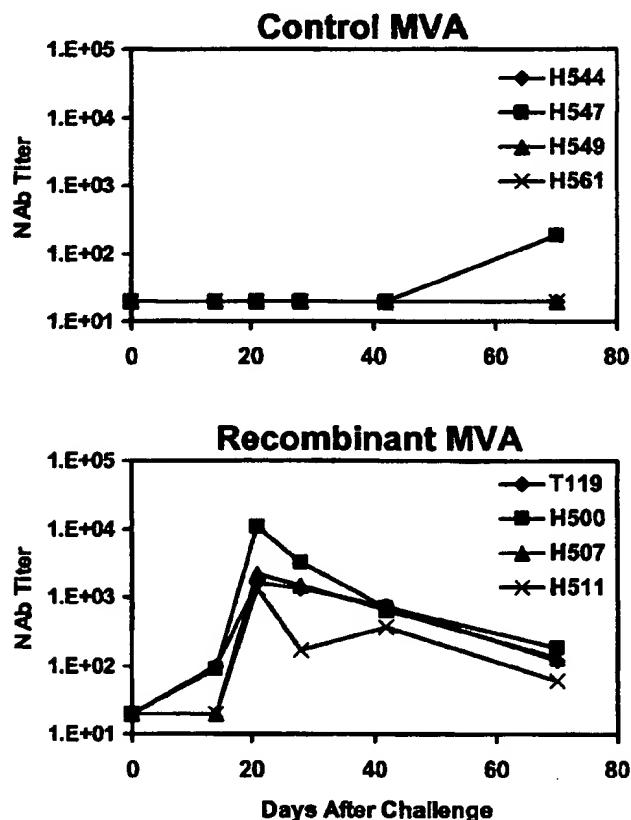
<sup>a</sup> SIV Gag p11C-specific CD8<sup>+</sup> T-cell responses as measured by tetramer staining of freshly isolated and peptide-stimulated PBMC and chromium release functional cytotoxicity assays at day 17 after challenge. The percent CD3<sup>+</sup> CD8<sup>+</sup> cells that bind tetramer is shown for the tetramer assays. The percent specific lysis at a 5:1 effector-to-target ratio is shown for the cytotoxicity assays.

CD4<sup>+</sup> T lymphocytes. In the vaccinated animals, monkeys H500 and H511 had completely stable CD4<sup>+</sup> T-lymphocyte counts, whereas monkeys T119 and H507 exhibited partial declines in CD4<sup>+</sup> T-lymphocyte counts by day 168 after chal-

lenge. On day 70 after challenge, a time by which the setpoint of viral replication is reached in SHIV-89.6P-infected rhesus monkeys, the CD4<sup>+</sup> T-lymphocyte counts in the vaccinated monkeys were significantly higher than in the control monkeys ( $P = 0.028$  by a two-sided Wilcoxon rank sum test).

We next measured plasma viral RNA levels in the monkeys by a real-time amplification assay with a detection limit of 500 copies/ml (17, 39). As demonstrated in Fig. 5, the control monkeys developed high levels of peak primary viremia, reaching  $5.4 \times 10^7$  to  $3.8 \times 10^8$  copies/ml on day 10 or 14 after challenge. In the vaccinated monkeys, peak primary viremia was slightly lower, between  $4.4 \times 10^6$  and  $1.4 \times 10^8$  copies/ml. On day 70 after challenge, all the control animals had high-setpoint viral RNA levels of  $1.2 \times 10^4$  to  $5.9 \times 10^5$  copies/ml. In three of the four vaccinated monkeys (T119, H500, and H511), setpoint viremia was below the limit of detection of the assay (<500 copies/ml). Setpoint viremia in monkey H507, however, remained high. Interestingly, this animal had the lowest levels of vaccine-elicited CTLs prior to challenge. A trend toward a reduction in setpoint viremia was observed in the vaccinated animals compared with the control monkeys ( $P = 0.11$  by a two-sided Wilcoxon rank sum test). The vaccinated animals had a 2.0-log reduction in geometric mean viral RNA levels after setpoint compared with the control animals.

## A. SHIV-89.6



## B. SHIV-89.6P

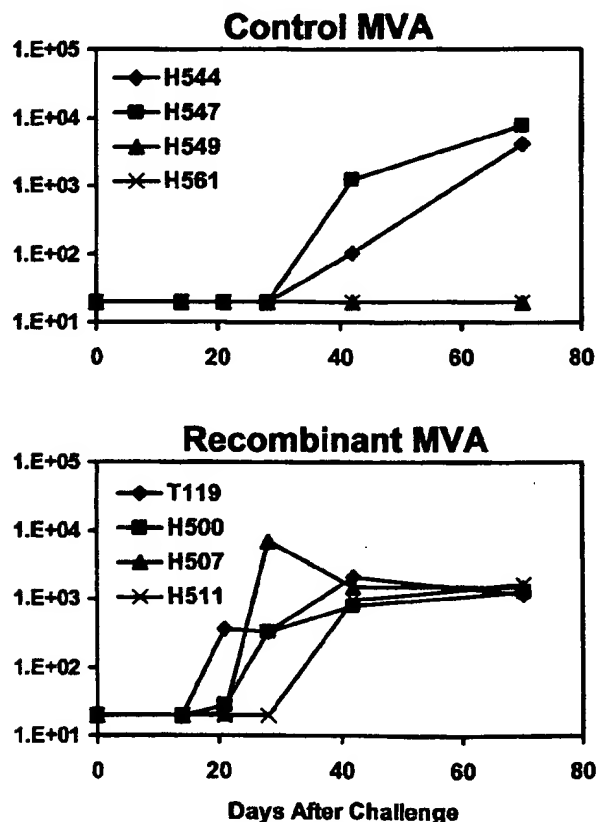


FIG. 3. NAb responses following challenge. Plasma antibody titers capable of neutralizing SHIV-89.6 (A) and SHIV-89.6P (B) were measured by MT-2 cell-killing assays at multiple time points (9).

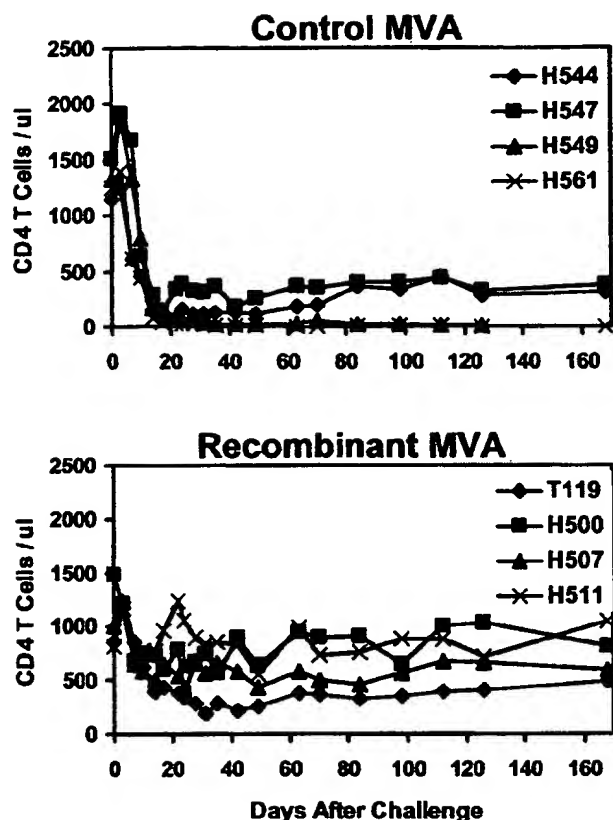


FIG. 4. CD4<sup>+</sup> T-lymphocyte counts following challenge. CD4<sup>+</sup> T-lymphocyte counts in peripheral blood were determined by multiplying the total lymphocyte count by the percentage of CD3<sup>+</sup> CD4<sup>+</sup> lymphocytes at multiple time points.

Significant clinical disease progression was observed in the two control animals (H549 and H561) that had complete depletion of their CD4<sup>+</sup> T lymphocytes, persistent high viremia, and no SHIV-89.6P-specific NAb. These two animals died at days 126 and 168 after challenge. In contrast, all the vaccinated animals remained healthy without evidence of clinical disease or mortality by day 168 after challenge. The rapid development of clinical AIDS and mortality in the control animals is comparable with our previous experience with SHIV-89.6P-infected monkeys (7, 34).

**Immune correlates of protection.** A scatter plot of data shown in Fig. 6 demonstrates a significant correlation between prechallenge vaccine-elicited plateau-phase p11C-specific CTL responses determined by tetramer staining and day 70 postchallenge setpoint viral RNA levels ( $P = 0.03$  by a two-sided Spearman rank correlation test). This correlation is analogous to the correlation we observed in our recent study of immune responses and the protective efficacy elicited by DNA vaccination (7).

## DISCUSSION

In this study, virus-specific immune responses were elicited in rhesus monkeys using recombinant MVA vectors expressing

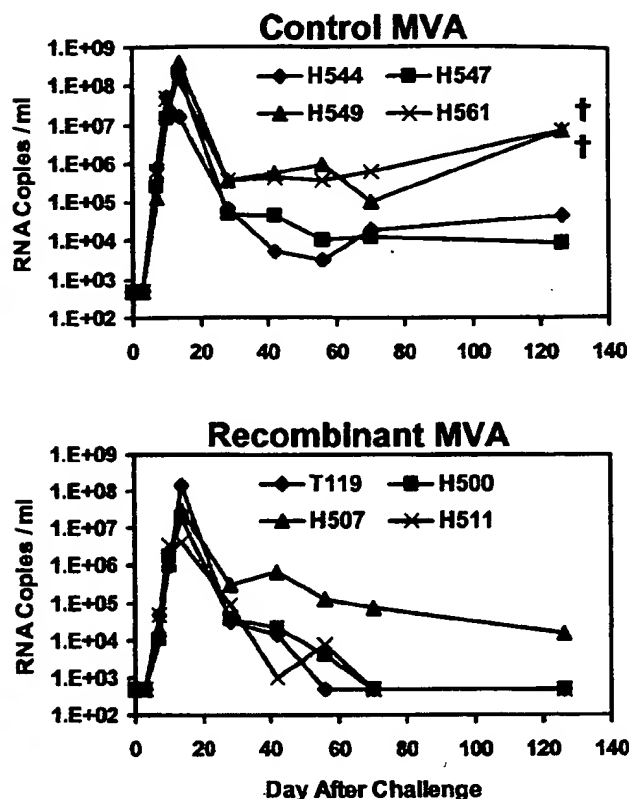


FIG. 5. Viral RNA levels following challenge. Plasma viral RNA levels were determined at multiple time points by a real-time amplification assay with a detection limit of 500 copies/ml (17, 39). †, death of the animal.

SIV *gag-pol* and HIV-1 89.6 *env*. The kinetics and magnitude of the vaccine-elicited SIV Gag epitope-specific CTL responses were comparable to those observed in our previous study of recombinant MVA-vaccinated rhesus monkeys (37, 38). The levels of vaccine-elicited CTL responses in the present study were also comparable to those elicited by plasmid DNA vaccination in our prior studies, but were lower than those elicited by cytokine-augmented DNA vaccination (6, 7, 12).

As we reported previously (7), there was a statistically sig-

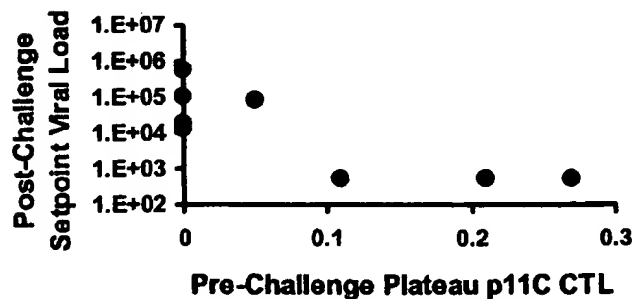


FIG. 6. Correlation of prechallenge vaccine-elicited plateau-phase p11C-specific CTL responses as determined by tetramer staining and day 70 postchallenge setpoint viral RNA levels ( $P = 0.03$ ).

nificant correlation between levels of prechallenge vaccine-elicited plateau-phase CTLs and setpoint viremia following challenge. The asymptotic appearance of the data from the scatter plots in both of these studies suggests that a level of vaccine-elicited plateau-phase CTLs may exist above which little additional benefit is obtained after challenge. The prechallenge plateau-phase CTL population presumably represents the vaccine-elicited memory pool of CTLs which expand upon exposure to virus to become functional effector CTLs. The correlation observed between levels of prechallenge plateau-phase CTLs and the control of viremia following challenge highlights the importance of CTLs in controlling AIDS virus replication.

Following the SHIV-89.6P challenge, secondary SIV Gag epitope-specific CTL responses were clearly evident in the vaccinated animals. The secondary CTL responses were maximal on day 14 and then rapidly declined to steady-state plateau levels. The magnitude of the secondary CTL responses following viral challenge reflected both the levels of vaccine-elicited CTL responses as well as the levels of viral antigen driving these responses. The vaccinated monkey that was unable to control viremia (H507) had persistently high levels of tetramer-binding CD8<sup>+</sup> T cells following challenge, likely reflecting the high levels of antigen present in this animal.

NAbs specific for SHIV-89.6 and SHIV-89.6P were undetectable in the vaccinated monkeys at the time of peak vaccine-elicited immunity or on the day of challenge. However, high-titer SHIV-89.6-specific NAb were detected in the vaccinated animals within 3 weeks after challenge. Since SHIV-89.6-specific NAb are rarely detected in naïve animals prior to 6 weeks following infection with SHIV-89.6 or SHIV-89.6P (9, 24), the SHIV-89.6-specific NAb response observed here was most likely an anamnestic response primed by the vaccine. This secondary NAb response following challenge was presumably elicited by either shared epitopes between the Env 89.6 immunogen and the Env 89.6P on the challenge virus or a minor SHIV-89.6 quasispecies present in the SHIV-89.6P challenge stock.

NAb specific for SHIV-89.6P were detected by day 21 to 42 after challenge in the vaccinated monkeys and considerably later or not at all in the control monkeys. It is unclear if the earlier emergence of SHIV-89.6P-specific NAb in the vaccinated animals reflected de novo generation of NAb facilitated by the preserved CD4<sup>+</sup> T-cell help in these animals, affinity maturation of the SHIV-89.6-specific NAb, or a secondary NAb response that was primed by the vaccine. This last possibility is perhaps least likely, since SHIV-89.6-specific NAb have poor neutralizing activity against SHIV-89.6P (9, 25). Regardless of the mechanism, these data demonstrate that the rapid emergence of NAb responses specific for a highly pathogenic primary isolate-like challenge virus did not require immunization with a completely homologous Env construct.

On day 14 after challenge, at the time of peak primary viremia, secondary CTL responses were maximal and SHIV-89.6P-specific NAb were undetectable, suggesting that the initial control of primary viremia in the vaccinated animals was mediated predominantly by CTLs. The subsequent control of viremia, however, likely reflects the effects of both cellular and humoral immune responses. In our prior study utilizing MVA vectors expressing *env* smH-4 and the related challenge virus

SIVsmE660, the monkeys vaccinated with MVA/*env* developed secondary SIVsmH-4-specific NAb after SIVsmE660 challenge but no convincing secondary SIVsmE660-specific NAb (28, 29). It is possible that the absence of augmented SIVsmE660-specific NAb following challenge accounted for the observation that MVA/*gag-pol*, MVA/*env*, and MVA/*gag-pol-env* vaccinations all provided comparable partial control of viremia in that study.

A significant limitation of the present study is the small number of monkeys, which precluded a statistical comparison of clinical disease end points and mortality. However, following the SHIV-89.6P challenge, the control animals developed low-frequency CTL responses, low-titer NAb responses, rapid loss of CD4<sup>+</sup> T lymphocytes, high viral RNA levels, and clinical disease and death in two of the four animals in this group. The monkeys that received the recombinant MVA vaccines developed high-frequency CTL responses, high-titer NAb responses, partial preservation of CD4<sup>+</sup> T lymphocytes, reduced viral RNA levels, and no evidence of clinical disease or mortality by day 168 after challenge. The 2.0-log reduction in mean setpoint plasma viremia observed in this study is similar to the 1.9-log reduction we have reported in SHIV-89.6P-challenged monkeys using plasmid DNA vaccination, although it is less striking than the 3.0-log reduction achieved using cytokine-augmented DNA vaccination (7). The results of the present study are also comparable with the results we obtained with recombinant MVA vaccination in conjunction with an SIVsmE660 challenge (37, 38).

The degree of protection achieved against SHIV-89.6P-induced AIDS by recombinant MVA vaccination and plasmid DNA vaccination should not be interpreted as evidence that SHIV-89.6P-induced disease is easy to ameliorate. In fact, several other vaccine modalities, including purified recombinant proteins and synthetic peptide vaccines, provide no discernible protection against SHIV-89.6P viremia or clinical disease progression in similarly conducted vaccine trials with rhesus monkeys (N. L. Letvin et al., unpublished data). The fact that SHIV-89.6P infection rapidly leads to immunodeficiency and AIDS in the majority of control monkeys makes this a useful challenge model for assessing the ability of vaccine candidates to provide protection against clinical disease progression in a relatively short time frame.

It is likely that a number of vaccine approaches will ultimately prove to have comparable efficacy in eliciting immune responses, controlling viremia, and preventing clinical sequelae of an AIDS virus infection. Such approaches are likely to include recombinant live vectors (38), plasmid DNA (7), and prime-boost approaches that involve boosting a DNA-primed immune response with a recombinant live vector (2, 15, 19, 35). Many of these vaccine strategies will be tested for their utility in humans over the next several years. If viral replication is similarly reduced in vaccinated humans who are subsequently infected with HIV-1, such individuals may demonstrate slowed disease progression and decreased HIV-1 transmission rates (30). Thus, a vaccine that elicits immunity that is not sterilizing but capable of reducing HIV-1 RNA levels following infection may still provide substantial clinical benefits to human populations.

## ACKNOWLEDGMENTS

We thank Russell Byrum, Bioqual Inc., and Simoy Goldstein for generous assistance.

This study was supported in part by NIH grants AI-85343 and AI-26507.

## REFERENCES

- Allen, T. M., J. Sidney, M.-F. del Guercio, R. L. Glickman, G. L. Lensmeyer, D. A. Wiebe, R. DeMars, C. D. Pauza, R. P. Johnson, A. Sette, and D. I. Watkins. 1998. Characterization of the peptide binding motif of a rhesus MHC class I molecule (Mamu-A\*01) that binds an immunodominant CTL epitope from SIV. *J. Immunol.* 160:6062-6071.
- Allen, T. M., T. U. Vogel, D. H. Fuller, B. R. Mothe, S. Steffen, J. E. Boyson, T. Shipley, J. Fuller, T. Hanke, A. Sette, J. D. Altman, B. Moss, A. J. McMichael, and D. I. Watkins. 2000. Induction of AIDS virus-specific CTL activity in fresh, unstimulated peripheral blood lymphocytes from rhesus macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regimen. *J. Immunol.* 164:4968-4978.
- Altman, J. D., P. A. H. Moss, P. J. R. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274:94-96.
- Baba, T. W., Y. S. Jeong, D. Penninck, R. Bronson, M. F. Greene, and R. M. Ruprecht. 1995. Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* 267:1820-1825.
- Baba, T. W., V. Liska, A. H. Khimani, N. B. Ray, P. J. Dailey, D. Penninck, R. Bronson, M. F. Greene, H. M. McClure, L. N. Martin, and R. M. Ruprecht. 1999. Live attenuated, multiple deleted simian immunodeficiency virus causes AIDS in infant and adult macaques. *Nat. Med.* 5:194-203.
- Barouch, D. H., A. Craiu, M. J. Kuroda, J. E. Schmitz, X. X. Zheng, S. Santra, J. D. Frost, G. R. Krivulka, M. A. Lifton, C. L. Crabbs, G. Heidecker, H. C. Perry, M.-E. Davies, H. Xie, C. E. Nickerson, T. D. Steenbeke, C. I. Lord, D. C. Montefiori, T. B. Strom, J. W. Shiver, M. G. Lewis, and N. L. Letvin. 2000. Augmentation of immune responses to HIV-1 and simian immunodeficiency virus DNA vaccines by IL-2/Ig plasmid administration in rhesus monkeys. *Proc. Natl. Acad. Sci. USA* 97:4192-4197.
- Barouch, D. H., S. Santra, J. E. Schmitz, M. J. Kuroda, T.-M. Fu, W. Wagner, M. Bilska, A. Craiu, X. X. Zheng, G. R. Krivulka, K. Beaudry, M. A. Lifton, C. E. Nickerson, W. L. Triglona, K. Punt, D. C. Freed, L. Guan, S. Dubey, D. Casimiro, A. Simon, M.-E. Davies, M. Chastain, T. B. Strom, R. S. Gelman, D. C. Montefiori, M. G. Lewis, E. A. Emini, J. W. Shiver, and N. L. Letvin. 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* 290:486-492.
- Belyakov, I. M., L. S. Wyatt, J. D. Ahlers, P. Earl, C. D. Pendleton, B. L. Kelsall, W. Strober, B. Moss, and J. A. Berzofsky. 1998. Induction of a mucosal cytotoxic T-lymphocyte response by intrarectal immunization with a replication-deficient recombinant vaccinia virus expressing human immunodeficiency virus 89.6 envelope protein. *J. Virol.* 72:8264-8272.
- Crawford, J. M., P. L. Earl, B. Moss, K. A. Reimann, M. S. Wyand, K. H. Manson, M. Bilska, J. T. Zhou, C. D. Pauza, P. W. H. I. Parren, D. R. Burton, J. G. Sodroski, N. L. Letvin, and D. C. Montefiori. 1999. Characterization of primary isolate-like variants of simian-human immunodeficiency virus. *J. Virol.* 73:10199-10207.
- Daniel, M. D., F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers. 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* 258:1938-1941.
- Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Ellett, C. Chatfield, V. A. Lawson, S. Crowe, A. Maerz, S. Sonza, J. Learmont, J. S. Sullivan, A. Cunningham, D. Dwyer, D. Dowton, and J. Mills. 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* 270:988-991.
- Egan, M. A., W. A. Charini, M. J. Kuroda, G. Voss, J. E. Schmitz, P. Racz, K. Tenner-Racz, K. Manson, M. Wyand, M. A. Lifton, C. E. Nickerson, T. M. Fu, J. W. Shiver, and N. L. Letvin. 2000. Simian immunodeficiency virus (SIV) gag DNA-vaccinated rhesus monkeys develop secondary cytotoxic T-lymphocyte responses and control viral replication after pathogenic SIV infection. *J. Virol.* 74:7485-7495.
- Egan, M. A., M. J. Kuroda, G. Voss, J. E. Schmitz, W. A. Charini, C. I. Lord, M. A. Forman, and N. L. Letvin. 1999. Use of major histocompatibility complex class I/peptide/β2m tetramers to quantitate CD8<sup>+</sup> cytotoxic T lymphocytes specific for dominant and nondominant viral epitopes in simian-human immunodeficiency virus-infected rhesus monkeys. *J. Virol.* 73:5466-5472.
- Greenough, T. C., J. L. Sullivan, and R. C. Desrosiers. 1999. Declining CD4 T-cell counts in a person infected with nef-deleted HIV-1. *New Engl. J. Med.* 340:236-237.
- Hanke, T., R. V. Samuel, T. J. Blanchard, V. C. Neumann, T. M. Allen, J. E. Boyson, S. A. Sharpe, N. Cook, G. L. Smith, D. I. Watkins, M. P. Cranage, and A. J. McMichael. 1999. Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multi-epitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. *J. Virol.* 73:7524-7532.
- Hirsch, V. M., T. R. Fuerst, G. Sutter, M. W. Carroll, L. C. Yang, S. Goldstein, M. Piatak, W. Elkins, W. G. Alvord, D. C. Montefiori, B. Moss, and J. D. Lifson. 1996. Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *J. Virol.* 70:3741-3752.
- Igarashi, T., Y. Endo, G. Englund, R. Sadjadpour, T. Matano, C. Buckler, A. Buckler-White, R. Plishka, T. Theodore, R. Shibata, and M. Martin. 1999. Emergence of a highly pathogenic simian/human immunodeficiency virus in a rhesus macaque treated with anti-CD8 mAb during a primary infection with a nonpathogenic virus. *Proc. Natl. Acad. Sci. USA* 96:14049-14054.
- Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho. 1999. Dramatic rise in plasma viremia after CD8<sup>+</sup> T cell depletion in simian immunodeficiency virus-infected macaques. *J. Exp. Med.* 189:991-998.
- Kent, S. J., A. Zhao, S. J. Best, J. D. Chandler, D. B. Boyle, and I. A. Ramshaw. 1998. Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. *J. Virol.* 72:10180-10188.
- Knapp, L. A., E. Lehmann, M. S. Fiekarczyk, J. A. Urvater, and D. I. Watkins. 1997. A high frequency of Mamu-A\*01 in the rhesus macaque detected by polymerase chain reaction with sequence-specific primers and direct sequencing. *Tissue Antigens* 50:657-661.
- Kuroda, M. J., J. E. Schmitz, D. H. Barouch, A. Craiu, T. M. Allen, A. Sette, D. I. Watkins, M. A. Forman, and N. L. Letvin. 1998. Analysis of Gag-specific cytotoxic T lymphocytes in simian immunodeficiency virus-infected rhesus monkeys by cell staining with a tetrameric major histocompatibility complex class I-peptide complex. *J. Exp. Med.* 187:1373-1381.
- Learmont, J. C., A. F. Geczy, J. Mills, L. J. Ashton, C. H. Raynes-Greenow, R. J. Garsia, W. B. Dyer, L. McIntyre, R. B. Oelrichs, R. I. Rhodes, N. J. Deacon, and J. S. Sullivan. 1999. Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. *New Engl. J. Med.* 340:1715-1722.
- Meyer, H., G. Sutter, and A. Mayr. 1991. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *J. Gen. Virol.* 72:1031-1038.
- Miller, M. D., H. Yamamoto, A. H. Hughes, D. I. Watkins, and N. L. Letvin. 1991. Definition of an epitope and MHC class I molecule recognized by Gag-specific cytotoxic T lymphocytes in SIVmac-infected rhesus monkeys. *J. Immunol.* 147:320-329.
- Montefiori, D. C., K. A. Reimann, M. S. Wyand, K. Manson, M. G. Lewis, R. G. Collman, J. G. Sodroski, D. P. Bolognesi, and N. L. Letvin. 1998. Neutralizing antibodies in sera from macaques infected with chimeric simian-human immunodeficiency virus containing the envelope glycoproteins of either a laboratory-adapted variant or a primary isolate of human immunodeficiency virus type 1. *J. Virol.* 72:3427-3431.
- Musey, L., J. Hughes, T. Schacker, T. Shea, L. Corey, and M. J. McElrath. 1997. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. *New Engl. J. Med.* 337:1267-1274.
- Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, A. Hurley, M. Markowitz, D. D. Ho, D. F. Nixon, and A. J. McMichael. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279:2103-2106.
- Oourmanov, I., M. Bilska, V. M. Hirsch, and D. C. Montefiori. 2000. Recombinant modified vaccinia virus Ankara expressing the surface gp120 of simian immunodeficiency virus (SIV) primes for a rapid neutralizing antibody response to SIV infection in macaques. *J. Virol.* 74:2960-2965.
- Oourmanov, I., C. R. Brown, B. Moss, M. Carroll, L. Wyatt, L. Pletneva, S. Goldstein, D. Venzon, and V. M. Hirsch. 2000. Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV. *J. Virol.* 74:2740-2751.
- Quinn, T. C., M. J. Wawer, N. Sewankambo, D. Serwadda, C. Li, F. Wabwire-Mangen, M. O. Meehan, T. Lutalo, and R. H. Gray. 2000. Viral load and heterosexual transmission of human immunodeficiency virus type 1. *New Engl. J. Med.* 342:921-929.
- Redfield, R. R., D. C. Wright, W. D. James, T. S. Jones, C. Brown, and D. S. Burke. 1987. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. *New Engl. J. Med.* 316:673-676.
- Reimann, K. A., J. T. Li, G. Voss, C. Lekutis, K. Tenner-Racz, P. Racz, W. Lin, D. C. Montefiori, D. E. Lee-Parritz, Y. Lu, R. G. Collman, J. Sodroski, and N. L. Letvin. 1996. An env gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. *J. Virol.* 70:3198-3206.
- Reimann, K. A., J. T. Li, R. Vazey, M. Halloran, L.-W. Park, G. B. Karlsson,

- J. Sodroski, and N. L. Letvin. 1996. A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate *env* causes an AIDS-like disease after in vivo passage in rhesus monkeys. *J. Virol.* 70:6922-6928.
34. Reimann, K. A., A. Watson, P. J. Dailey, W. Lin, C. L. Lord, T. D. Steenbeke, R. A. Parker, M. K. Axthelm, and G. B. Karlsson. 1999. Viral burden and disease progression in rhesus monkeys infected with chimeric simian-human immunodeficiency viruses. *Virology* 256:15-21.
  35. Robinson, H. L., D. C. Montefiori, R. P. Johnson, K. H. Manson, M. L. Kalish, J. D. Lifson, T. A. Rizvi, S. Lu, S.-L. Hu, G. P. Mazzara, D. L. Panicali, J. G. Herndon, R. Glickman, M. A. Candido, S. L. Lydy, M. S. Wyand, and H. M. McClure. 1999. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nat. Med.* 5:526-534.
  36. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifson, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8<sup>+</sup> lymphocytes. *Science* 283:857-860.
  37. Seth, A., I. Ourmanov, M. J. Kuroda, J. E. Schmitz, M. W. Carroll, L. S. Wyatt, B. Moss, M. A. Forman, V. M. Hirsch, and N. L. Letvin. 1998. Recombinant modified vaccinia virus Ankara-simian immunodeficiency virus gag pol elicits cytotoxic T lymphocytes in rhesus monkeys detected by a major histocompatibility complex class I/peptide tetramer. *Proc. Natl. Acad. Sci. USA* 95:10112-10116.
  38. Seth, A., I. Ourmanov, J. E. Schmitz, M. J. Kuroda, M. A. Lifson, C. E. Nickerson, L. Wyatt, M. Carroll, B. Moss, D. Venzon, N. L. Letvin, and V. M. Hirsch. 2000. Immunization with a modified vaccinia virus expressing simian immunodeficiency virus (SIV) Gag-Pol primes for an anamnestic Gag-specific cytotoxic T-lymphocyte response and is associated with reduction of viremia after SIV challenge. *J. Virol.* 74:2502-2509.
  39. Suryanarayana, K., T. A. Wiltout, G. M. Vasques, V. M. Hirsch, and J. D. Lifson. 1998. Plasma SIV RNA viral load determination by real-time quantification of product generation in reverse transcriptase-polymerase chain reaction. *AIDS Res. Hum. Retrovir.* 14:183-189.
  40. Wyand, M. S., K. H. Manson, M. Garcia-Moll, D. Montefiori, and R. C. Desrosiers. 1996. Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. *J. Virol.* 70:3724-3733.
  41. Wyand, M. S., K. Manson, M. D. C. Montefiori, J. F. Lifson, R. P. Johnson, and R. C. Desrosiers. 1999. Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. *J. Virol.* 73:8356-8363.
  42. Wyatt, L. S., S. T. Shors, B. R. Murphy, and B. Moss. 1996. Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. *Vaccine* 14:1451-1458.
  43. Wyatt, L. S., S. S. Whitehead, K. A. Venzon, B. R. Murphy, and B. Moss. 1999. Priming and boosting immunity to respiratory syncytial virus by recombinant replication-defective vaccinia virus MVA. *Vaccine* 18:392-397.

# Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations

HARRIET L. ROBINSON<sup>1</sup>, DAVID C. MONTEFIORI<sup>2</sup>, R. PAUL JOHNSON<sup>3</sup>, KELLEDY H. MANSON<sup>4</sup>,  
MARCIA L. KALISH<sup>5</sup>, JEFFREY D. LIFSON<sup>6</sup>, TAHIR A. RIZVI<sup>7</sup>, SHAN LU<sup>8</sup>, SHIU-LOK HU<sup>9</sup>,  
GAIL P. MAZZARA<sup>10</sup>, DENNIS L. PANICALI<sup>10</sup>, JAMES G. HERNDON<sup>1</sup>, RHONA GLICKMAN<sup>3</sup>,  
MARIA A. CANDIDO<sup>1</sup>, SHARI L. LYDY<sup>1</sup>, MICHAEL S. WYAND<sup>4</sup> & HAROLD M. MCCLURE<sup>1</sup>

<sup>1</sup>Yerkes Regional Primate Research Center, Atlanta, Georgia 30329, USA

<sup>2</sup>Duke University Medical Center, Durham, North Carolina 27710, USA

<sup>3</sup>New England Regional Primate Center and AIDS Research Center of Harvard Medical School,  
Southborough, Massachusetts, 01772, USA

<sup>4</sup>GTC-Mason Laboratories, Worcester, Massachusetts 01608, USA

<sup>5</sup>Centers for Disease Control, Atlanta, Georgia 30333, USA

<sup>6</sup>NCI-Frederick Cancer Research Center, Frederick, Maryland 21702, USA

<sup>7</sup>M.D. Anderson Cancer Center, University of Texas, Bastrop, Texas 78802, USA

<sup>8</sup>Division of Infectious Diseases, University of Massachusetts Medical Center,  
Worcester, Massachusetts 01655, USA

<sup>9</sup>Washington Regional Primate Research Center, University of Washington, Seattle, Washington 98195, USA

<sup>10</sup>Therion Biologics, Cambridge, Massachusetts 02142, USA

Correspondence should be addressed to H.L.R.

**Eight different protocols were compared for their ability to raise protection against immunodeficiency virus challenges in rhesus macaques. The most promising containment of challenge infections was achieved by intradermal DNA priming followed by recombinant fowl pox virus booster immunizations. This containment did not require neutralizing antibody and was active for a series of challenges ending with a highly virulent virus with a primary isolate envelope heterologous to the immunizing strain.**

With the prospect of HIV, first recognized in 1984, infecting 1% of the world's population by the year 2000, the need for the development of a vaccine for AIDS is urgent<sup>1</sup>. Challenges for an effective vaccine include preventing infection by a virus that is not easily blocked by neutralizing antibody<sup>2,3</sup>, containing a virus that preferentially kills the T-helper cells that support the amplification and differentiation of anti-viral immune responses<sup>4,5</sup>, and preventing the establishment of the reservoir of latent proviral DNA that can serve as a source of re-emergent virus<sup>6</sup>. These are difficult goals for a vaccine to achieve.

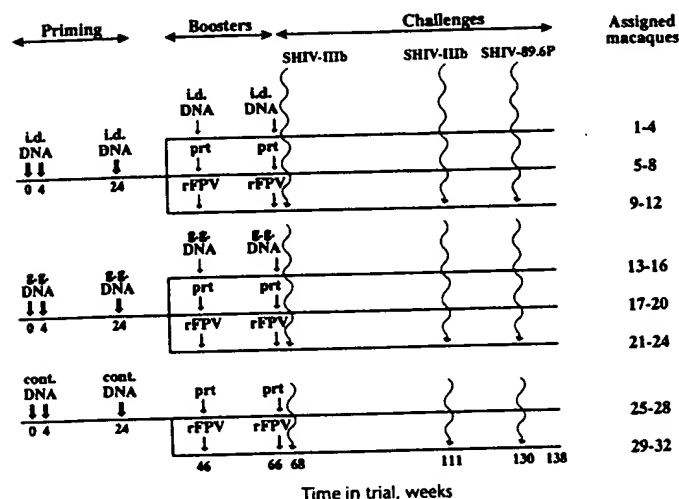
Among the AIDS vaccines that have been tested in macaque models, only live attenuated viruses with potential risks for disease have contained highly pathogenic challenges<sup>7-9</sup>. Three safer approaches for vaccination, including purified proteins, recombinant viral vectors and DNA vaccines, have held some promise in less-virulent challenge models. Purified envelope glycoproteins (Envs) have generated sterilizing immunity that correlated with the presence of neutralizing antibody. However the efficacy of this neutralizing activity has been limited by its poor persistence and its specificity for the immunizing Env, but not a spectrum of patient Envs (refs. 10-12). Live vectored vaccines, such as recombinant pox viruses expressing Env or capsid proteins (Gag) have raised both antibody and cell-mediated immune responses<sup>13-15</sup>. For live vectors, the expression of both Env and Gag has raised better containment than the expression of only Env or

Gag<sup>16</sup>. The third approach, DNA vaccines, also raises both humoral and cell-mediated immune responses<sup>17,18</sup>. DNA vaccines have contained a highly attenuated HIV-1 infection in chimpanzees (HIV-1-SF2)(ref. 19), and achieved sterilizing immunity when the DNA vaccine was followed by a protein booster and challenge was with a virus with an Env homologous to the booster Env protein<sup>20</sup>.

We undertook this study to gain experience with the use of DNA for an immunodeficiency virus vaccine and to evaluate the ability of DNA, or DNA followed by protein or recombinant pox virus booster immunizations, to raise protection. Would vaccines that were providing some success when used individually have synergy when used together? Our results show that intradermal DNA priming followed by recombinant pox virus boosters can contain challenge infections below the level of detection of our PCR assays for viral RNA. This apparently cell-mediated containment did not require detectable levels of neutralizing antibody.

## Vaccine trial and immunogens

The vaccine trial had eight groups with four rhesus macaques in each (Fig 1). Two methods of DNA delivery were evaluated: intradermal injections of DNA in saline, and gene gun bombardments of DNA-coated gold beads. A popular method of DNA delivery, intramuscular inoculation, was not used, because of overlap with other NIH sponsored trials. Groups 1-3 tested intra-

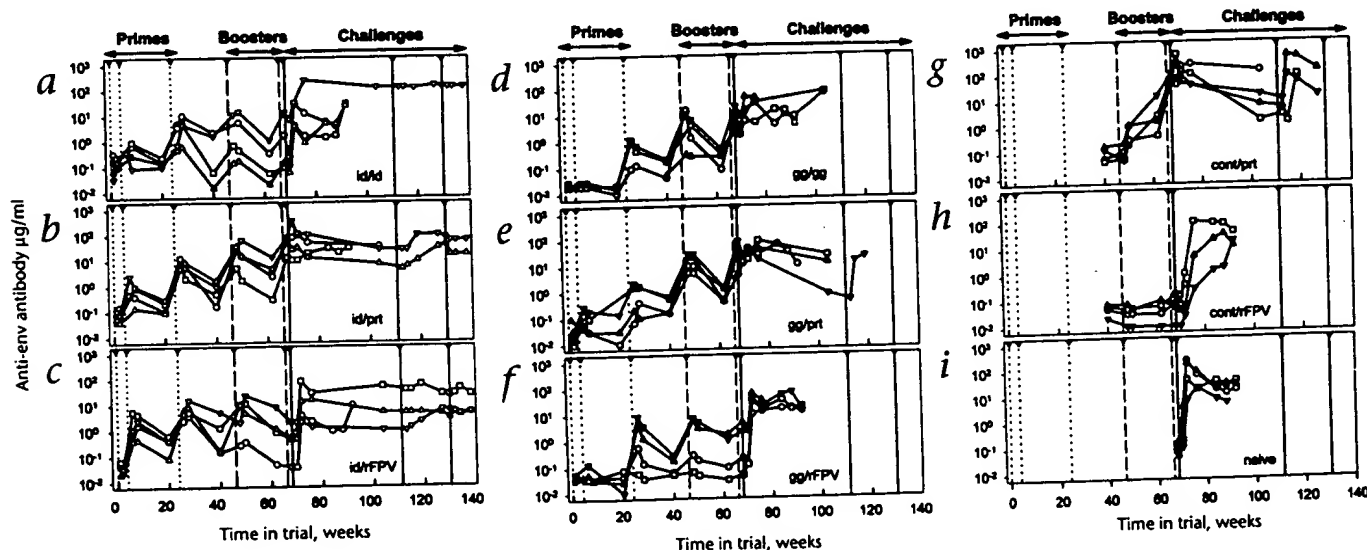


**Fig. 1** Vaccine trial. Downward arrows, timing for priming and booster immunizations and challenge infections for the eight sections of the trial. Far right, monkeys assigned to each group. i.d., intradermal; g.g., gene gun; DNA, five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant; rFPV, three recombinant fowl pox viruses; cont. DNA, vaccine vector without an insert.

dermal priming with DNA followed by booster immunizations with intradermal DNA inoculations, purified Env protein or recombinant fowl pox virus (rFPV). Groups 4–6 evaluated priming with DNA delivered by gene gun followed by booster immunizations with DNA delivered by gene gun, Env protein or rFPV. Groups 7 and 8 tested priming with control DNA (plasmid vector without a vaccine insert) followed by boosting with Env protein or rFPV. The DNA 'primers' were administered at 0, 4 and 26 weeks and the booster immunizations, at 46 and 66 weeks. Intravenous challenges were with chimeras between simian and human immunodeficiency viruses (SHIVs) that allow infection of rhesus macaques with viruses with HIV-1 Envs. The first two

challenges were with SHIV-IIIb, the virus from which the immunogens had been derived. SHIV-IIIb is a nonpathogenic virus that grows to lower titers in macaques than typical primary isolates achieve in humans<sup>21,22</sup>. The third challenge was with SHIV-89.6P, a very virulent virus with the envelope glycoprotein of a primary isolate<sup>23</sup>. In rhesus macaques, SHIV-89.6P causes the depletion of CD4<sup>+</sup> T cells within 2 weeks of infection and death from opportunistic infections within 6 months.

The DNA and rFPV immunogens were designed to express the *gag*, *pol*, *env* and *nef* genes of SHIV-IIIb. Five DNA constructs were used for each DNA inoculation: The first vaccine DNA, pRS102, encoded the Gag and polymerase proteins of SIVmac239; the second, pCMV/nef, encoded the Nef protein of SIVmac239; the third, pJW4303/HXB-2d pol, was designed to express non-infectious HIV-1-IIIb virus-like-particles; the fourth, pJW4303/HXB-2.gp140, encoded a secreted oligomeric form of the HIV-1-IIIb Env that is unstable and sheds gp120; and the fifth, pJW4303/HXB-2.gp120, encoded a secreted monomeric form of the receptor binding subunit of the HIV-1-IIIb Env (gp120). A combination of Env-expressing plasmids was used because a similar combination had proved effective at raising neutralizing antibody in another DNA vaccine trial<sup>24</sup>. For intradermal immunizations, 500 µg of each DNA were administered for a total of 2.5 mg of DNA per inoculation. For the gene gun inoculations, 4 µg of each DNA was delivered for a total of 20 µg of DNA per inoculation. Less DNA is required to raise immune responses by gene gun administration than by saline injections of DNA because of the relatively efficient transfection that results from the direct delivery of DNA into cells by DNA-coated gold bullets<sup>25,26</sup>. The protein boost consisted of 100 µg of the full-length, unmutated Env of HIV-1-IIIb (ref. 27) in incomplete Freund's adjuvant administered intramuscularly. The IIIb Env had an apparent molecular weight of 160 kDa with gp120 and gp41 covalently attached. Recombinant fowl pox was chosen for the pox virus boost because fowl pox infects but does not replicate in macaques, posing no risk of unwanted dissemination of the vector in the vaccinated monkey. Three SHIV-IIIb-expressing rFPVs



**Fig. 2** Temporal antibody responses during the immunization and challenge phases of the vaccine trial. Titers of IgG specific for Env were determined by ELISA. Each panel presents data for one group of the trial. Lower right corners, priming immunizations/booster immunizations: i.d., intradermal delivery of five vaccine DNAs; g.g., gene gun delivery of five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant;

rFPV, three recombinant fowl pox virus vectors; cont., plasmid vector without a vaccine insert. Symbols indicate order in group: O, first monkey in each group (macaques 1, 5, 9, 13, 17, 21, 25, 29, 33, 37 and 39); □, second (2, 6, 10, 14, 18, 22, 26, 30, 34, 38 and 40); Δ, third (7, 11, 15, 19, 23, 27, 31, 35 and 41); ▽, fourth (4, 8, 12, 16, 20, 24, 28, 32 and 36).

Table 1 Specific CTL activity generated in different groups of the trial

Trial group, macaque number		Time in trial							
		6 weeks, 2 weeks after second prime	20 weeks, 4 weeks before third prime	26 weeks, 2 weeks after third prime	40 weeks, 6 weeks before first boost	48 weeks, 2 weeks after first boost	62 weeks, 4 weeks before second boost day of first challenge	68 weeks, 2 weeks after second boost	111 weeks, 43 weeks after first challenge
i.d./i.d.	1	-/-*	-/-	-/-	-/-	1/-		-/-	
	2	-/-	-/-	-/-	-/-	-/-		-/-	
	3	-/-	-/-	-/-	1/-	-/1/2		-/-	
	4	-/-	-/-	-/-	-/-	-/-		-/-	1/1-
i.d./prt	5	-/-	-/-	-/-	-/-	-/-		-/-	1/1-
	6	-/-	-/-	-/2/2	-/-	-/-		-/-	
	7	-/-	-/-	1/-	2/1-	-/-		-/-	
	8	-/-	-/-	-/-	-/-	-/-		-/-	-/-
i.d./rFPV	9	-/-	-/-	-/-	-/-	-/-		1/-/1	
	10	2/-	-/-	-/-	2/1/2	-/1		-/-	
	11	-/-	-/-	-/-	-/-	1/-		1/-	2/2-
	12	-/-	-/-	1/-	2/2/2	1/2/3		1/-	2/-
g.g./g.g.	13	1/-	-/-	-/-	1/-	-/-	-/-	-/-	4/3/1
	14	1/-	-/-	1/-	-/-	1/1-	-/-	-/-	
	15	-/1	-/-	-/-	-/-	1/1/1	-/-	-/-	
	16	-/-	-/-	-/1-	-/-	1/-	1/-	-/-	2/-
g.g./prt	17	-/-	2/-	-/-	1/-/1	1/-/2	-/1	-/-	
	18	-/-	-/-	-/-	-/-	1/-	-/-	-/-	2/1-
	19	-/-	-/-	-/-	-/1-	-/-	-/1	-/-	1/1/1
	20	-/-	-/-	-/-	-/-	-/1-	-/-	-/-	-/-
g.g./rFPV	21	2/-	2/-	1/-	-/-	1/-	-/2	-/-	
	22	2/5-	-/-	-/-	-/-	2/1/1	1/-	-/-	
	23	2/-	2/-	-/-	-/-	3/4-	-/-	-/-	3/1-
	24	-/2	-/2	-/-	1/-	1/-	2/-	1/-	
cont/prt	25			-/- <sup>b</sup>			-/-	-/-	-/1-
	26			-/-			-/-	-/-	-/-
	27			-/-			-/-	-/-	-/-
	28			-/-			-/-	-/-	-/-
cont/rFPV	29			-/- <sup>b</sup>			-/-	-/-	
	30			-/-			-/-	-/-	
	31			-/-			-/-	-/-	
	32			-/-			-/-	-/-	

CTL responses were measured after 2 weeks of re-stimulation. Herpes papio-immortalized B cell lines and recombinant vaccinia viruses expressing Gag, Env or Nef were used for restimulation and for targets<sup>29</sup>. Spontaneous release of target cells was <25% in all assays. Effector:target ratios for which background lysis of control vaccinia virus-expressing cells exceeded 20% were excluded from analysis. Based on examination of naive monkeys, specific lysis of greater than 5% observed at more than one effector:target ratio was interpreted as significant. Three effector to target ratios were used in assays (40:1, 20:1 and 10:1). \*Scores for specific lysis for Gag/Env/Nef targets. -, <5%; 1, 5-10%; 2, 10-20%; 3, 20-30%; 4, 30-40%; 5, 40-50% specific lysis. In concurrent CTL assays on PBMCs from macaques infected with live-attenuated viruses, CTL assays consistently scored higher than 2. <sup>b</sup>Assays done at week 34. i.d., intradermal delivery of five vaccine DNAs; g.g., gene gun delivery of five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant; rFPV, three recombinant fowl pox virus vectors; cont., plasmid vector without a vaccine insert.

included one expressing Gag-Pol, one expressing Env, and one expressing Nef (ref. 28). Of each rFPV,  $5 \times 10^8$  PFU were administered intramuscularly, for a total dose of  $1.5 \times 10^9$  PFU.

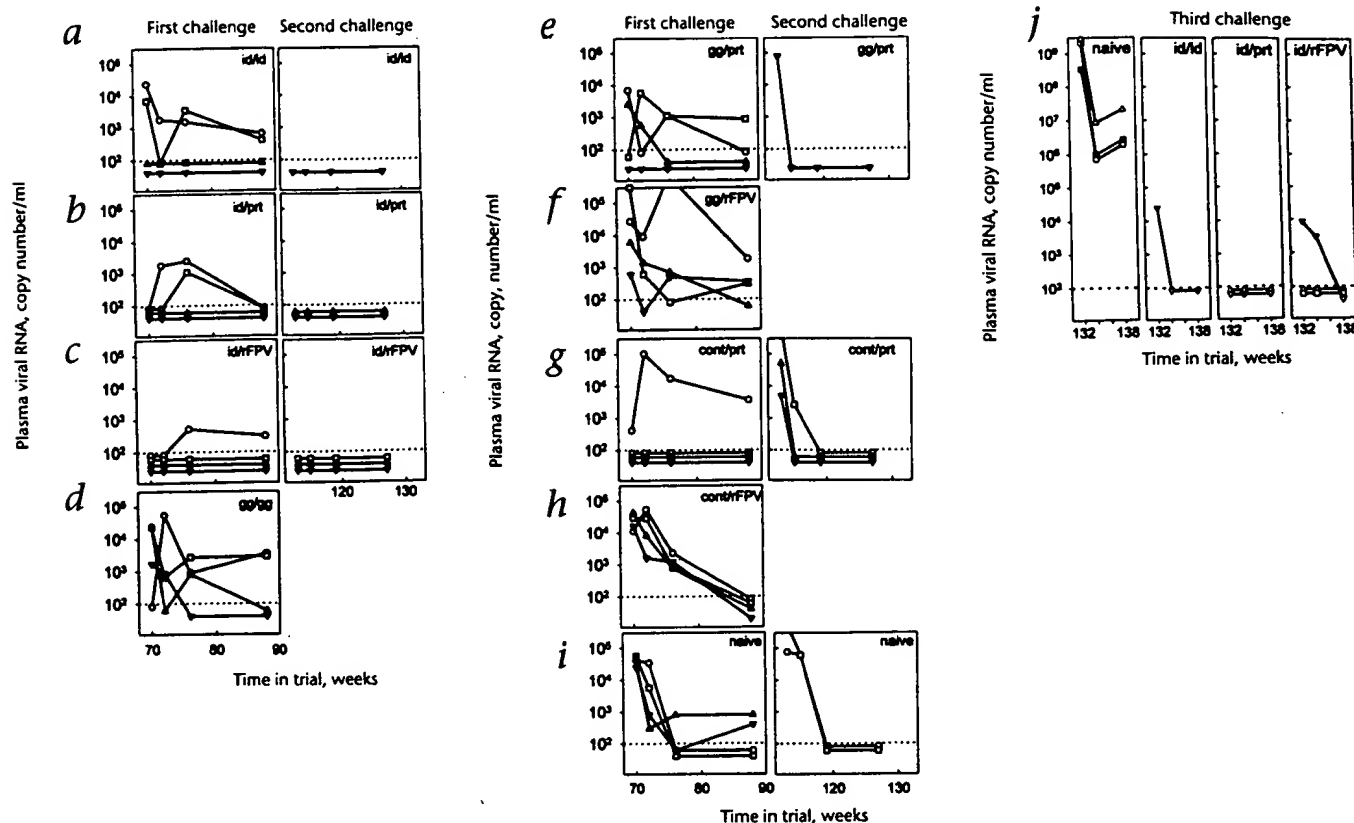
#### Antibody and CTL responses to the immunogens

The different DNA priming and booster inoculations generated distinct patterns of antibody responses (Fig. 2). During the priming phase of the immunizations, the intradermal DNA inoculations generated antibodies against Env that appeared earlier and rose to higher titers than the gene gun-generated antibody (geometric mean titers of 3.5 and 0.47  $\mu$ g per ml, respectively;  $P = 0.01$ , Mann-Whitney U test). The fourth and fifth intradermal DNA boosters failed to increase the levels of antibody above the peak primed response (Fig. 2a), whereas the gene gun DNA boosters increased specific antibody to a geometric mean titer of 6.1  $\mu$ g per ml (Fig. 2d). The protein boosters generated the highest titers of antibody achieving geometric mean titers of 43, 58 and 71  $\mu$ g per ml, or 20-30% of the level of antibody to Env pre-

sent in a pooled serum from rhesus macaques chronically infected with SHIV-IIIb. The rFPV boosters were the least successful booster immunization for antibody. For the monkeys primed with DNA delivered intradermally, the first, but not the second rFPV booster, increased the levels of falling antibody (Fig. 2c). For the monkeys primed with DNA delivered by gene gun, low level increases in falling responses were observed for each of the rFPV boosts (Fig. 2f). Undetectable levels of anti-Env IgG were raised in the group receiving control DNA plus rFPV (Fig. 2h).

The frequency and activity of specific cytolytic T lymphocytes (CTLs) were low throughout the trial (Table 1). Of the 156 assays for CTLs, only 70 were positive. In these positive assays, Gag was a target in 39 assays; Nef, in 17; and Env, in 14. Analysis of a subset of monkeys demonstrated that the CTL activity was MHC-restricted and mediated by CD8<sup>+</sup> cells (data not shown). As an index for CTL activity, scores were assigned that reflected the presence and extent of CTL lysis; these scores were summed over time for the monkeys within a group. In the four assays before





**Fig. 3** Levels of viral RNA in plasma after the first, second and third challenges. Temporal levels of plasma viral RNA were determined using real-time PCR (ref. 31). **a–i**, first and second challenges; **j**, third challenge. Upper right corners, priming immunizations/booster immunizations: i.d., intradermal delivery of five vaccine DNAs; g.g., gene gun delivery of five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant; rFPV, three recombinant fowl

pox virus vectors; cont., plasmid vector without a vaccine insert. Symbols indicate order in group: ○, first monkey in each group (macaques 1, 5, 9, 13, 17, 21, 25, 29, 33, 37 and 39); □, second (2, 6, 10, 14, 18, 22, 26, 30, 34, 38 and 40); △, third (7, 11, 15, 19, 23, 27, 31, 35 and 41); ▽, fourth (4, 8, 12, 16, 20, 24, 28, 32 and 36). Levels of viral RNA below detection were scored as 100; limit of detection, 300–1,000 copies of viral RNA per ml of plasma.

the booster immunizations, specific cytolytic activity for the monkeys primed with DNA delivered by gene gun had a cumulative score of 32, compared with 23 for the monkeys primed with DNA delivered intradermally. At 2 weeks after the first booster, groups receiving the rFPV booster had the highest scores. After the second rFPV booster immunization, the lytic scores were lower than those after the first boosters. On the day of the first challenge, only four monkeys had low levels of specific CTL activity (<10% lysis). These included three macaques in the group primed with DNA delivered intradermally and boosted with rFPV virus, and one monkey in the group primed with DNA delivered by gene gun and boosted with rFPV. No monkey receiving control DNA vaccination had a positive CTL response at any time before challenge (data not shown). Concurrent assays for cytolytic activity using peripheral blood mononuclear cells (PBMCs) from macaques infected with live attenuated viruses consistently showed intermediate to high levels of lytic activity<sup>29</sup>. Thus, the levels of vaccine-raised CTLs were lower and less consistent than those seen for live attenuated SIVs.

#### First and second challenges: SHIV-IIIb

The first two challenges were with SHIV-IIIb, a virus homologous to the immunogens. Ten macaque infectious doses of SHIV-IIIb were administered intravenously at 2 weeks after the second booster immunization, a time when peak titers of neutralizing antibody were expected to be present<sup>30</sup>. Protection was evaluated

by measuring virion-associated SIV RNA in plasma using a quantitative real-time RT-PCR (ref. 31) and by determining the frequency of PBMCs that could initiate an infection when co-cultivated with CEMx174 cells, a very susceptible cell line<sup>32</sup>. Monkeys were given subsequent challenges if plasma viral RNA could not be detected at any time after challenge and if only one co-cultivation assay showed low levels of infected cells. After the first challenge, ten monkeys met these criteria for protection and were given a second SHIV-IIIb challenge 43 weeks after the first challenge. No booster immunization was given before the second challenge to allow assessment of the long term efficacy of the different vaccine protocols against the homologous challenge.

Unexpectedly, the intradermal DNA priming provided better containment of the SHIV-IIIb challenge than the gene gun DNA priming ( $P = 0.01$ , Fisher exact test) (Fig. 3). All four of the naive control monkeys had levels of plasma viral RNA greater than  $1 \times 10^4$  copies/ml. Six of the twelve monkeys primed with DNA delivered intradermally had undetectable levels of plasma viral RNA after the first two challenges, whereas all of the monkeys primed with DNA delivered by gene gun had easily detected levels of viral RNA after the first or second challenge. The best containment occurred in the group primed with DNA delivered intradermally and boosted with rFPV (Fig. 3c). After the first challenge, three members of this group had undetectable levels of plasma viral RNA, and the fourth had very low levels of viral RNA. Three of the monkeys receiving control DNA plus Env pro-

**Table 2** Detection of infection after challenge: cumulative levels of plasma viral RNA and cumulative number of co-cultivation-positive PBMCs

Group/ macaque		First SHIV-IIIb challenge		Second SHIV-IIIb challenge		Third SHIV89.6P challenge	
		Viral RNA, copies <sup>a</sup>	Co-cultivation-positive cells <sup>c</sup>	Viral RNA, copies	Co-cultivation-positive cells	Viral RNA, copies	Co-cultivation-positive cells
naive <sup>a</sup>		76,000	770	649,000	149	$2.6 \times 10^9$	83,455
		61,700	743	135,000	8,751	$0.3 \times 10^9$	ND
		45,900	177,178			$2.7 \times 10^9$	ND
		27,200	6,615				
id/i.d	1	26,950	6578				
	2	10,800	252				
	3	<	41				
i.d./prt	4	<	<	<	<	24,000	27
	5	4,300	29				
	6	1,100	14				
i.d./rFPV	7	<	<	<	9	< <sup>d</sup>	<
	8	<	<	<	9	< <sup>d</sup>	<
	9	800	41				
	10	<	14	<	<	< <sup>d</sup>	9
	11	<	9	<	<	< <sup>d</sup>	<
	12	<	<	<	<	11,600	56
g.g./g.g.	13	58,400	< <sup>e</sup>				
	14	29,250	14 <sup>e</sup>				
	15	24,800	243				
g.g./prt	16	2,550	14 <sup>e</sup>				
	17	9,300	81				
	18	7,750	< <sup>e</sup>				
	19	3,050	14				
	20	<	< <sup>e</sup>	78,000	49		
g.g./rFPV	21	727,800	490				
	22	30,900	246				
	23	8,100	13				
cont/prt	24	1,450	246				
	25	121,000	14				
	26	<	14	752,600	29,426		
	27	<	<	51,000	136		
	28	<	<	4,900	27		
cont/rFPV	29	66,300	406				
	30	57,850	3,281				
	31	51,750	732				
	32	18,800	1,098				

<sup>a</sup>Naive macaques are not numbered because different macaques were used to control for the three challenges. <sup>b</sup>Sum of the titers of plasma viral RNA/ml at 2, 4, 8 and 20 weeks after the first challenge; 2, 4, 8 and 16 weeks after the second challenge; and 2, 4 and 8 weeks after the third challenge. <, below the background for detection. Backgrounds for detection, 300–1,000. <sup>c</sup>Sum of the co-cultivation-positive cells per  $1 \times 10^6$  PBMCs at 2, 4, 8 and 12 weeks after the first challenge; 2, 4 and 8 weeks after the second challenge; and 2 and 4 weeks after the third challenge. <sup>e</sup>, monkeys for which data for 4 weeks after challenge were not available. <, no co-cultivation-positive cells detected in  $1 \times 10^6$  PBMCs. <sup>d</sup>Monkeys given the transfection test for detection of occult virus (Table 3). ND, not done; i.d., intradermal delivery of five vaccine DNAs; g.g., gene gun delivery of five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant; rFPV, three recombinant fowl pox virus vectors; cont., plasmid vector without a vaccine insert.

tein also had undetectable levels of viral RNA after the first challenge. However, all of these were infected by the second challenge (Fig. 3g). None of the monkeys receiving control DNA plus rFPV were protected against the first challenge (Fig. 3h).

Assays for co-cultivation positive cells and anamnestic antibody responses showed that monkeys that did not have detectable plasma viral RNA by PCR assay had sustained low levels of infection. After the first challenge, four of the eleven monkeys with undetectable levels of viral RNA had detectable levels of co-cultivation-positive cells and anamnestic antibody responses (Table 2 and Fig. 2). Three additional monkeys had detectable levels of anamnestic antibody responses (Fig. 2a, macaque  $\nabla$ ; macaques  $\Delta$  and  $\nabla$ ). After the second challenge, two of the six monkeys with undetectable levels of viral RNA had low levels of co-cultivation-positive cells and anamnestic antibody responses (Table 2 and Fig. 2), and a third had an increase in antibody levels (Fig. 2c, macaque  $\nabla$ ).

### Third challenge: SHIV-89.6P

The six monkeys that did not have detectable levels of plasma viral RNA after the first two challenges were challenged 19 weeks later with the highly virulent SHIV-89.6P virus. Two weeks after the SHIV-89.6P challenge, the naive control monkeys had undergone a precipitous decrease in their numbers of CD4<sup>+</sup> T cells (data not shown) and had very high levels of plasma viral RNA (Fig. 3j). All of the monkeys given the third challenge were protected against the rapid loss of CD4<sup>+</sup> cells (data not shown). Four did not have detectable levels of viral RNA by PCR assay (Fig. 3j). Three of these did not have detectable levels of co-cultivation-positive cells (Table 2). The two monkeys that did have detectable levels of viral RNA by PCR assay had titers 0.001% those of the naive control monkeys. By 8 weeks after challenge, these two monkeys had reduced their levels of viral RNA to below background, whereas levels of viral RNA of greater than  $1 \times 10^6$  copies/ml persisted in the naive control monkeys.

### Neutralizing antibody

Neutralizing antibody was present at different titers in the different sections and phases of the trial (Fig. 4). On the day of the first challenge, low-to-undetectable levels of neutralizing antibody were present in the group primed with DNA delivered intradermally and boosted with rFPV. In contrast, in the group primed with control DNA and boosted with Env protein, neutralizing antibody approximated the titer of 200, which is considered a correlate of protein-raised protection against SHIV-IIIb (ref. 33). In the groups primed with vaccine DNAs and boosted with protein or DNA, titers of neutralizing antibody ranged from intermediate to low. For monkeys given the second challenge, titers of neutralizing antibody against SHIV-IIIb on the day of challenge were both higher and lower than those on the day of the first challenge for the three groups of monkeys primed with DNA delivered intradermally, but were consistently lower for the monkey primed with DNA delivered by gene gun and boosted with protein, and monkeys receiving control DNA and protein boosters. On the day of the third challenge, none of the monkeys remaining in the trial had detectable levels of neutralizing antibody for SHIV-89.6P.

### Transfusion test

At 8 weeks after the third challenge, 10 ml of whole blood from the four macaques with undetectable levels of infection (Table 2, macaques 7, 8, 10 and 11), were transfused into naive macaques to see if an occult infection could be transmitted (Table 3). Not unexpectedly, macaque 10, which had co-cultivation-positive cells at two weeks after the SHIV-89.6P challenge transmitted SHIV-89.6P. However, unexpectedly, macaque 7 that had neither detectable levels of viral RNA nor co-cultivation-positive cells for SHIV-89.6P transmitted the earlier SHIV-IIIb challenge virus. This macaque had transient low levels of co-cultivation-positive cells (9 cells per  $1 \times 10^6$  PBMCs) at 2 weeks after the second challenge but never had detectable levels of plasma virus RNA (Table 2). Macaque 8, which transiently had co-cultivation-positive cells for SHIV-IIIb after the second challenge, did not transmit SHIV-IIIb. Macaque 11, which transiently had co-cultivation-positive cells for SHIV-IIIb after the first challenge, also did not transmit SHIV-IIIb.

### Discussion

We tested eight different protocols for vaccination against immunodeficiency viruses and found one protocol that contained challenge infections in the absence of detectable levels of neutralizing antibody. This very promising protocol consisted of intradermal DNA priming followed by rFPV booster immunizations (Fig. 3 and Table 2). The presumably cellular immune responses that mediated protection were at levels close to those considered 'background' in our assays for CTLs (Table 1), and were well below the level of CTLs in concurrent assays conducted on rhesus macaques infected with live attenuated SIVs (ref. 29). This raises the possibility that the protective response might have been mediated by a nonlytic T-cell activity. Two types of T-cell-mediated anti-immunodeficiency virus responses have been reported: a cytolytic, MHC-I-restricted activity, and a nonlytic, secreted activity that suppresses HIV-1 infections<sup>34,35</sup>.

Intradermal DNA priming was more effective than gene gun DNA priming in raising protective responses ( $P = 0.01$ ). This was seen in the groups receiving DNA

and Env protein boosters as well as in the groups boosted with rFPV (Fig. 3 and Table 2). In the three groups primed with DNA delivered intradermally, six of twelve monkeys contained the first two challenges below the level that could be detected by RT-PCR assays for plasma viral RNA. In contrast, none of the monkeys primed with DNA delivered by gene gun were similarly protected. This difference in efficacy did not manifest itself in before-challenge assays for CTL or antibody (Table 1 and Figs. 2 and 4) and would not have been realized in the absence of the challenges. One manifestation of the difference in efficacy was lower levels of plasma viral RNA per co-cultivation-positive cell in the monkeys primed with DNA delivered intradermally than in the groups primed with DNA delivered by gene gun ( $P < 0.03$ , Mann-Whitney U test) (Table 2). This could reflect the possibility that intradermal but not gene gun DNA inoculations prime T-cell responses with suppressive activity for HIV-1 infections.

Assuming results can be extrapolated from murine to primate models, the difference in the efficacy of the responses primed with DNA delivered intradermally and by gene gun may be due to saline injections of DNA priming predominantly type 1 T-cell help, and gene gun deliveries of DNA priming predominantly type 2 T-cell help<sup>36</sup>. Differences in the type of a primed T-cell response are maintained during subsequent booster immunizations or challenge infections<sup>36</sup>. Type 1 and type 2 T-helper cells express different patterns of lymphokines and chemokines and are associated with distinctive CTLs (refs. 37,38). Differences in these two types of immune responses could have profound effects on the ability of cell-mediated immune responses to contain immunodeficiency virus challenges.

Different aspects of the immune response seemed to play different parts in the control of the immunodeficiency virus challenges in the different sections of the trial and for the different challenges within a section. This is consistent with multiple aspects of immune responses having the potential to contribute to protective immunity<sup>39</sup>. At the time of the first challenge, an apparently cell-mediated protection (in the absence of detectable levels of neutralizing antibody) occurred in the rFPV-boosted group of the monkeys primed with DNA delivered intradermally (Fig. 4). In the groups not boosted with rFPV, containment was more likely in monkeys that had intermediate-to-high levels of neutralizing antibody than in monkeys with low titers of neutralizing antibody ( $P < 0.04$ , Mann-Whitney U test) (Fig. 4). At the time of the second challenge, the titers of neutralizing antibody had decreased in the one monkey primed with DNA delivered by gene gun and boosted

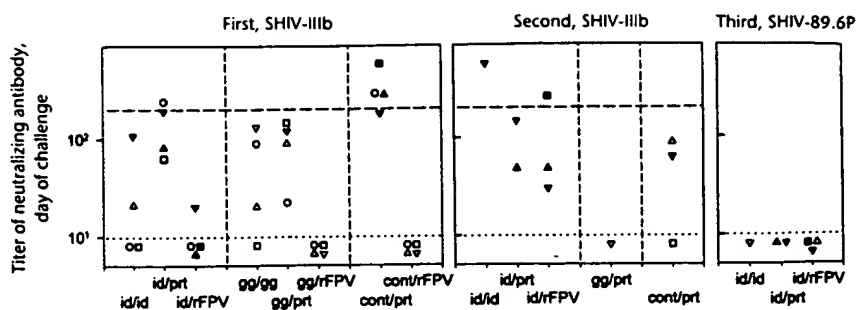
**Table 3** Use of blood transfusions to test for the presence of infection in the protected macaques

Donor macaque	History of after-challenge virus recovery for donor, (RT-PCR/co-cultivation)			Infection of recipient	Proviral V3 region of infecting virus*
	First IIIb	Second IIIb	Third 89.6P		
vaccinated					
7	-/-	-/+	-/-	yes	SHIV-IIIb
8	-/-	-/+	-/-	no	-
10	-/+	-/-	-/+	yes	SHIV89.6P
11	-/+	-/-	-/-	no	-
Control 39			+/+	yes	SHIV-89.6P

\* -, failure to detect proviral DNA in PBMCs by PCR. IIIb, SHIV-IIIb; 89.6P, SHIV-89.6P.

**Fig. 4** Titers of neutralizing antibody for the challenge virus on the days of the first, second and third challenges. Neutralizing antibody responses are the reciprocal of the serum dilution giving 50% neutralization of SHIV-IIIb (a and b) or SHIV-89.6P (c) using a cell-killing assay and neutral red staining<sup>39</sup>. Symbols indicate order in group: ○, first monkey in each group (macaques 1, 5, 9, 13, 17, 21, 25, 29, 33, 37 and 39); □, second (2, 6, 10, 14, 18, 22, 26, 30, 34, 38 and 40); △, third (7, 11, 15, 19, 23, 27, 31, 35 and 41); ▽, fourth (4, 8, 12, 16, 20, 24, 28, 32 and 36); Open symbols, infected monkeys; filled symbols, monkeys with strongly contained infections.

Horizontal dotted lines, the limit of detection for the assay; horizontal dashed lines, titer of neutralizing antibody that correlates with protection against the SHIV-IIIb challenge in SHIV-IIIb Env protein-immunized monkeys<sup>33</sup>. Below graphs, priming immunizations/booster immuniza-



tions: i.d., intradermal delivery of five vaccine DNAs; g.g., gene gun delivery of five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant; rFPV, three recombinant fowl pox virus vectors; cont., plasmid vector without a vaccine insert.

with protein and in the three monkeys primed with control DNA and boosted with protein, which had contained the first challenge. All four of these monkeys were infected by the second challenge virus. However, monkeys primed with DNA delivered intradermally that had titers of neutralizing antibody overlapping those of the monkeys primed with control DNA and boosted with protein contained the second challenge, and were presumably protected by cell-mediated responses as well as neutralizing antibody. By the third challenge, a neutralizing antibody-independent, presumably cell-mediated containment of the SHIV-89.6P challenge seemed to be occurring in monkeys from all three of the groups primed with DNA delivered intradermally (Fig. 4 and Table 2).

In the monkeys primed with DNA delivered intradermally, protective cell-mediated immune responses are likely to have been boosted by the challenge infections (much as they were boosted by the rFPV infection). These protective boosters were not observed in the groups primed with gene gun DNA or control DNA. This may reflect the live virus challenge boosting protective cell-mediated responses only in those groups in which protective cell-mediated responses had been primed. The putative booster immunizations differed from infection with SHIV-IIIb in their very low levels of virus replication. Infection of naive monkeys with SHIV-IIIb resulted in 27,000–649,000 copies of viral RNA and 149–177,000 co-cultivation-positive cells (Table 2). By contrast, the putative SHIV-IIIb boosters were accomplished with levels of infection that did not have detectable levels of plasma viral RNA and had  $\leq 14$  co-cultivation-positive cells (Table 2). These levels of infection are below the threshold of infection required for protection by live attenuated vaccines<sup>40</sup> and would not have provided containment in the absence of a vaccine-primed immune response.

The raising of protective cell-mediated responses by DNA primes and pox virus booster immunizations has precedence in studies in mice and macaques<sup>41–43</sup>. In these studies, the DNA prime was hypothesized to focus the immune response on the desired antigens, whereas the recombinant pox virus booster immunization was hypothesized to boost this response, both by the expression of higher levels of the recombinant antigen and the immunostimulatory activity of a pox virus infection. Our results contrast with those of a study in which gene gun DNA priming and rFPV booster immunizations protected three pig-tail macaques against an HIV-1 challenge<sup>43</sup>. In this highly attenuated infection (a maximum of 300 copies of viral RNA in

the naive control monkeys), gene gun DNA priming plus rFPV boosting may be able to afford protection against an immunodeficiency virus challenge.

Unlike antibody that can prevent infection, cell-mediated immune responses act after infection, and thus afford immunodeficiency virus infections the opportunity to establish proviral DNA. A transfection test at the end of the trial showed an otherwise undetected SHIV-IIIb infection in one of four monkeys tested (Table 3). In this monkey, the last SHIV-IIIb challenge had been 27 weeks before the transfusion. This monkey had never tested positive for plasma viral RNA and had shown low levels of co-cultivation-positive cells only once, at 25 weeks before the transfusion (Tables 2 and 3). This result indicates that HIV-1 vaccines that operate after infection, despite even very low levels of infection (below those able to be detected by RT-PCR), will not prevent the long term persistence of virus and the potential for re-emergent virus<sup>44</sup>.

The ability to achieve stringent containment of serial SHIV challenges using intradermal DNA priming and recombinant pox virus boosters holds promise for the development of a vaccine capable of considerably reducing viral replication and thus stemming the transmission of AIDS. This vaccine would be effective against a diversity of isolates because of its ability to raise neutralizing antibody-independent containment of infections. If our hypotheses are correct, the boosting of responses by challenge infections should bolster the effectiveness of the vaccine in the high-risk populations, which are the chief source of transmission.

## Methods

**Vaccine trial.** For intradermal immunizations, 500  $\mu$ g of each DNA was administered in four 100- $\mu$ l inoculations in the skin of the upper back for a total of 20 injections and 2.5 mg DNA per time point. For the gene gun inoculations, 4  $\mu$ g of each DNA was bombarded at 350 psi in eight shots, each containing 0.5 mg of gold, into shaved abdominal skin, for a total of 20  $\mu$ g and 40 gene gun deliveries of DNA per inoculation. The protein boost consisted of 100  $\mu$ g of the full-length Env of HIV-1-IIIb (ref. 27) in incomplete Freund's adjuvant, administered in two 0.5-ml inoculations, one in each biceps. Of each of three rFPV expressing Gag-Pol, Env or Nef (refs. 28,45),  $5 \times 10^8$  PFU were mixed in 1 ml and delivered in two 0.5-ml injections, one to each biceps, for a total dose of  $1.5 \times 10^9$  PFU. Care of the macaques was in accordance with institutional guidelines for the care and use of experimental animals.

**Immunogens.** A vaccine plasmid called pRS102 expressed SIVmac239 Gag and Pol proteins. The vaccine insert for pRS102 comprised a Kozak sequence, the SIV239 gag-pol region (nucleotides 1309–5753) and the

Mason-Pfizer Monkey virus cytoplasmic transport element. This insert was cloned into the *HindIII* and *NheI* sites of the eukaryotic expression vector pJW4303, and expression in transiently transfected COS cells was verified. pJW4303 was provided by J.I. Mullins (University of Washington, Seattle). A vaccine plasmid called pCMV/nef comprised the *PstI*-*StuI* Nef-encoding fragment of clone BK28 inserted into pCMV5 (ref. 46), and was provided by G. Viglianti (Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts). The pJW4303/HXB-2.dpol, pJW4303/HXB-2.gp140 and pJW4303/HXB-2.gp120 vaccine plasmids have been reported<sup>10</sup>.

**Assays for ELISA antibody.** The titers of anti-Env IgG in macaque sera were determined using an ELISA with affinity-purified sheep antibody against Env (catalog number 6205; International Enzymes, Fallbrook, California) to capture the gp120 form of a IIIB Env. Gp120 was produced using recombinant vaccinia virus vPE-50 (ref. 47). A standard curve was made using pooled sera from SHIV-IIIB-infected macaques whose anti-Env IgG concentration had been determined to be about 210 µg/ml. Sera were assayed at threefold dilutions in duplicate wells. Biotinylated anti-human IgG (H and L) and horse radish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, California) were used to detect bound IgG. Standard curves were fitted and sample concentrations were interpolated as µg per ml of sera using SOFTmax 2.3 software (Molecular Devices, Sunnyvale, California).

**Blood transfusion assay.** At 8 weeks after the third challenge, 10 ml of whole blood was transfused into naive recipients to test whether virus that could not be detected by co-cultivation or RT-PCR could be transmitted. Three naive monkeys challenged with SHIV-89.6P were used as positive controls. Transfusion recipients were tested over a 3-month period for infection by testing for 'antigenemia' and by co-cultivation of PBMCs with CEM-X174 cells at 0, 2, 4, 8 and 12 weeks after transfusion. The presence of virus was assayed using an antigen-capture ELISA for the p27 Gag protein of SIV (Coulter, Hialeah, Florida). The identity of viruses transmitted was tested by PCR amplifying and sequencing the C2V3C3 region of the proviral DNA in the PBMCs of recipient macaques<sup>48</sup>.

**Statistical analyses.** The Mann-Whitney U test was used to evaluate between-group differences in antibody titers or levels of plasma virus RNA per co-cultivation-positive cell. The Fisher exact test was used to examine differences in the frequency with which different trial groups contained viral infections. A type I error rate of 0.05 (two-tailed) was adopted as the criterion of significance for statistical inference.

#### Acknowledgments

We thank M. Feinberg and J. Safritz for critical comments on the manuscript. We are indebted to H. Drake-Perrow for administrative assistance. We thank M. Piatak, L. Li, and T. Parks for assistance with SIV RNA viral load analysis; J. Yang for assistance with T-cell assays; R. Schmidt for help in construction of pRS102; and A. Saekhou for assistance with analyses of transferred virus. This research was supported by NIH grants R01-AI-34241 and P01-AI-43045 (H.L.R.); R01-AI-40334 (S.L.); P01-AI-26503 (S.-L.H.) R01 AI 52634, RR 000138 (R. P. Johnson) and U01 AI 26507 (D.L.P.); by the Yerkes Primate Research Center Base Grant, RR-00165; and by contracts NCI-6S-1649 (D.C.M.) and NO1-CO-56000 (J.D.L.). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

RECEIVED 25 JANUARY; ACCEPTED 25 MARCH 1999

1. The World Health Organization in *AIDS Epidemic Update* (Joint United Nations Programme on HIV/AIDS and the World Health Organization, Geneva, 1998).
2. Moore, J.P. & Ho, D.D. HIV-1 neutralization: the consequences of viral adaptation to growth on transformed T cells. *AIDS* 9, 5117-136 (1995).
3. Burton, D.R. & Moore, J.P. Why do we not have an HIV vaccine and how can we make one? *Nature Med.* 4, 495-498 (1998).
4. Rosenberg, E.S. et al. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* 278, 1447-1450 (1997).
5. Schacker, T.W., Hughes, J.P., Shea, T., Coombs, R.W. & Corey, L. Biological and virologic characteristics of primary HIV infection. *Ann. Intern. Med.* 128, 613-620 (1998).
6. Finzi, D. & Siliciano, R.F. Viral dynamics in HIV-1 infection. *Cell* 93, 665-671 (1998).
7. Daniel, M.D., Kirchhoff, F., Czajak, S.C., Sehgal, P.K. & Desrosiers, R.C. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* 258, 1938-1941 (1992).
8. Baba, T.W. et al. Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* 267, 1820-1825 (1995).
9. Ruprecht, R.M., Baba, T.W. & Liska, V. Attenuated HIV vaccine: caveats. *Science* 271, 1790-1792 (1996).
10. Mascola, J.R. et al. Immunization with envelope subunit vaccine products elicits neutralizing antibodies against laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. The National Institute of Allergy and Infectious Diseases AIDS Vaccine Evaluation Group. *J. Infect. Dis.* 173, 340-348 (1996).
11. Connor, R.I. et al. Immunological and virological analyses of persons infected by human immunodeficiency virus type 1 while participating in trials of recombinant gp120 subunit vaccines. *J. Virol.* 72, 1552-1576 (1998).
12. Graham, B.S. et al. Analysis of intercurrent human immunodeficiency virus type 1 infections in phase I and II trials of candidate AIDS vaccines. AIDS Vaccine Evaluation Group, and the Correlates of HIV Immune Protection Group. *J. Infect. Dis.* 177, 310-319 (1998).
13. Moss, B. Recombinant DNA virus vectors for vaccination. *Semin. Immunol.* 2, 317-327 (1990).
14. Tartaglia, J. et al. Canarypox virus-based vaccines: prime-boost strategies to induce cell-mediated and humoral immunity against HIV. *AIDS Res. Hum. Retroviruses* 14 Suppl 3, S291-298 (1998).
15. Stott, J. & Hu, S.L. AIDS 1998. Vaccines and immunology: overview. *AIDS* 12, S95-96 (1998).
16. Hu, S.-L. et al. in *Transmembrane Protein and Core Antigens in Protection against SIV Infection* (eds. Chanock, R.M., Brown, F., Ginsberg, H.S. & Norrby, E.) 167-173 (Cold Spring Harbor Laboratory Press, New York, 1995).
17. Robinson, H.L. & Torres, C.A. DNA vaccines. *Semin. Immunol.* 9, 271-283 (1997).
18. Donnelly, J.J., Ulmer, J.B., Shiver, J.W. & Liu, M.A. DNA vaccines. *Annu. Rev. Immunol.* 15, 617-648 (1997).
19. Boyer, J.D. et al. Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination. *Nature Med.* 3, 526-532 (1997).
20. Letvin, N.L. et al. Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. *Proc. Natl. Acad. Sci. USA* 94, 9378-9383 (1997).
21. Li, J., Lord, C.I., Haseltine, W., Letvin, N.L. & Sodroski, J. Infection of cynomolgus monkeys with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoproteins. *J. Acquir. Immune Defic. Syndr.* 5, 639-646 (1992).
22. Li, J.T. et al. Persistent infection of macaques with simian-human immunodeficiency viruses. *J. Virol.* 69, 7061-7067 (1995).
23. Reimann, K.A. et al. A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. *J. Virol.* 70, 6922-6928 (1996).
24. Lu, S. et al. Simian immunodeficiency virus DNA vaccine trial in macaques. *J. Virol.* 70, 3978-3991 (1996).
25. Fynan, E.F. et al. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* 90, 11478-11482 (1993).
26. Pertmer, T.M. et al. Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine* 13, 1427-1430 (1995).
27. Kianiec, J. et al. Cross-neutralizing antibodies in rabbits immunized with HIV-1 gp160 purified from simian cells infected with a recombinant vaccinia virus. *AIDS Res. Hum. Retroviruses* 7, 791-798 (1991).
28. Jenkins, S. et al. Formation of lentivirus particles by mammalian cells infected with recombinant fowlpox virus. *AIDS Res. Hum. Retroviruses* 7, 991-998 (1991).
29. Johnson, R.P. et al. Induction of vigorous cytotoxic T-lymphocyte responses by live attenuated simian immunodeficiency virus. *J. Virol.* 71, 7711-8 (1997).
30. Richmond, J.F. et al. Studies of the neutralizing activity and avidity of anti-human immunodeficiency virus type 1 Env antibody elicited by DNA priming and protein boosting. *J. Virol.* 72, 9092-9100 (1998).
31. Suryanarayana, K., Wiltrout, T.A., Vasquez, G.M., Hirsch, V.M. & Lifson, J.D. Plasma SIV RNA viral load determination by real-time quantification of product generation in reverse transcriptase-polymerase chain reaction. *AIDS Res. Hum. Retroviruses* 14, 183-189 (1998).
32. Wyand, M.S., Manson, K.H., Garcia-Moll, M., Montefiori, D. & Desrosiers, R.C. Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. *J. Virol.* 70, 3724-3733 (1996).
33. Montefiori, D.C. & Evans, T.G. Toward an HIV-type 1 vaccine that generates potent, broadly cross-reactive neutralizing antibodies. *AIDS Res. and Hum. Retroviruses* 15, 689-698 (1999).
34. Yang, O.O. et al. Lysis of HIV-1-infected cells and inhibition of viral replication by universal receptor T cells. *Proc. Natl. Acad. Sci. USA* 94, 11478-11483 (1997).
35. Levy, J.A., Mackewicz, C.E. & Barker, E. Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of CD8+ T cells. *Immunol. Today* 17,

- 217–224 (1996).
36. Feltquate, D.M., Heaney, S., Webster, R.G. & Robinson, H.L. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J. Immunol.* **158**, 2278–84 (1997).
37. O'Garra, A. & Murphy, K. Role of cytokines in determining T-lymphocyte function. *Curr. Opin. Immunol.* **6**, 458–66 (1994).
38. Seder, R.A. & Paul, W.E. Acquisition of lymphokine-producing phenotype by CD4<sup>+</sup> T cells. *Annu. Rev. Immunol.* **12**, 635–673 (1994).
39. Dittmer, U., Brooks, D.M. & Hasenkrug, K.J. Requirement for multiple lymphocyte subsets in protection by a live attenuated vaccine against retroviral infection. *Nature Med.* **5**, 189–193 (1999).
40. Johnson, R.P. Live attenuated AIDS vaccines: hazards and hopes. *Nature Med.* **5**, 154–155 (1999).
41. Schneider, J. *et al.* Enhanced immunogenicity for CD8<sup>+</sup> T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nature Med.* **4**, 397–402 (1998).
42. Sedegah, M. *et al.* Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine. *Proc. Natl. Acad. Sci. USA* **95**, 7648–7653 (1998).
43. Kent, S.J. *et al.* Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. *J. Virol.* **72**, 10180–10188 (1998).
44. Polacino, P. *et al.* Limited breadth of the protective immunity elicited by simian immunodeficiency virus SIVmne gp160 vaccines in a combination immunization regimen. *J. Virol.* **73**, 618–630 (1999).
45. Gritz, L. *et al.* Generation of hybrid genes and proteins by vaccinia virus-mediated recombination: application to human immunodeficiency virus type 1 env. *J. Virol.* **64**, 5948–5957 (1990).
46. Andersson, S., Davis, D.L., Dahlback, H., Jornvall, H. & Russell, D.W. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264**, 8222–8229 (1989).
47. Earl, P.L. *et al.* Native oligomeric human immunodeficiency virus type 1 envelope glycoprotein elicits diverse monoclonal antibody reactivities. *J. Virol.* **68**, 3015–3026 (1994).
48. Schochetman, G., Subbarao, S. & Kalish, M.L. in *Viral Genome Methods* (ed. K.W. Adolph) 25–41 (CRC Press, Boca Raton, 1996).
49. Montefiori, D.C., Robinson, W.E.Jr., Schuffman, S.S. & Mitchell, W.M. Evaluation of antiviral drugs and neutralizing antibodies to human immunodeficiency virus by a rapid and sensitive microtiter infection assay. *J. Clin. Microbiol.* **26**, 231–235 (1988).

# Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques

IGOR M. BELYAKOV<sup>1</sup>, ZDENEK HEL<sup>2</sup>, BRIAN KELSALL<sup>3</sup>, VLADIMIR A. KUZNETSOV<sup>4</sup>, JEFFREY D. AHLERS<sup>1</sup>, JANOS NACSA<sup>2</sup>, DAVID I. WATKINS<sup>5</sup>, TODD M. ALLEN<sup>5</sup>, ALESSANDRO SETTE<sup>6</sup>, JOHN ALTMAN<sup>7</sup>, RUTH WOODWARD<sup>8</sup>, PHILLIP D. MARKHAM<sup>8</sup>, JOHN D. CLEMENTS<sup>9</sup>, GENOVEFFA FRANCHINI<sup>2</sup>, WARREN STROBER<sup>3</sup> & JAY A. BERZOFKY<sup>1</sup>

<sup>1</sup>Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch, National Cancer Institute, Bethesda, Maryland, USA

<sup>2</sup>Animal Model and Retroviral Vaccines Section, Basic Research Laboratory, National Cancer Institute, Bethesda, Maryland, USA

<sup>3</sup>Mucosal Immunity Section, Laboratory Of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA

<sup>4</sup>Section of Medical Biophysics, Laboratory of Integrative and Medical Biophysics, National Institute of Child Health and Human Development, Bethesda, Maryland, USA

<sup>5</sup>Wisconsin Regional Primate Research Center, Madison, Wisconsin, USA

<sup>6</sup>Epimmune, San Diego, California, USA

<sup>7</sup>Emory University Vaccine Center at Yerkes, Atlanta, Georgia, USA

<sup>8</sup>Advanced BioScience Laboratories, Kensington, Maryland, USA

<sup>9</sup>Department of Microbiology and Immunology, Program in Molecular Pathogenesis and Immunity, Tulane University School of Medicine, New Orleans, Louisiana, USA

Correspondence should be addressed to J.A.B.; email: [berzofsk@helix.nih.gov](mailto:berzofsk@helix.nih.gov) or I.M.B.; email: [belyakov@box-b.nih.gov](mailto:belyakov@box-b.nih.gov).

Given the mucosal transmission of HIV-1, we compared whether a mucosal vaccine could induce mucosal cytotoxic T lymphocytes (CTLs) and protect rhesus macaques against mucosal infection with simian/human immunodeficiency virus (SHIV) more effectively than the same vaccine given subcutaneously. Here we show that mucosal CTLs specific for simian immunodeficiency virus can be induced by intrarectal immunization of macaques with a synthetic-peptide vaccine incorporating the LT(R192G) adjuvant. This response correlated with the level of T-helper response. After intrarectal challenge with pathogenic SHIV-Ku2, viral titers were eliminated more completely (to undetectable levels) both in blood and intestine, a major reservoir for virus replication, in intrarectally immunized animals than in subcutaneously immunized or control macaques. Moreover, CD4<sup>+</sup> T cells were better preserved. Thus, induction of CTLs in the intestinal mucosa, a key site of virus replication, with a mucosal AIDS vaccine ameliorates infection by SHIV in non-human primates.

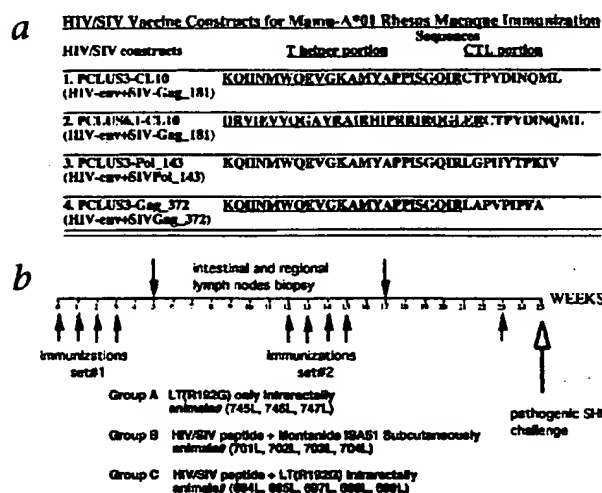
The mucosal surface is a major natural route of HIV-1 entry and the gut is a major site of HIV and simian immunodeficiency virus (SIV) replication<sup>1</sup>. Thus, a successful HIV/SIV vaccine will likely need to induce mucosal immunity<sup>2-5</sup>. Recent studies<sup>6-9</sup> of viral mucosal transmission indicate that cytotoxic T lymphocytes (CTLs) are an important component of the mucosal immune barrier. Therefore, we investigated whether CTLs generated in the gut mucosa by mucosal immunization may be more effective than CTLs generated elsewhere by systemic immunization.

In previous murine studies, we designed and tested prototype HIV synthetic-peptide vaccines consisting of a multideterminant peptide containing a cluster of overlapping helper epitopes (a cluster peptide)<sup>10</sup> colinearly synthesized with an epitope for neutralizing antibodies and CTLs (refs. 3,11). This peptide vaccine elicited neutralizing antibodies, CTLs and helper T cells<sup>12,13</sup> as did a similar vaccine studied by others in mice and monkeys<sup>14-16</sup>. This vaccine, with cholera toxin (CT) adjuvant, was more effective given intrarectally than intranasally or intragastrically in eliciting

both mucosal and systemic CTLs (ref. 2). Intrarectal immunization generated strong CTL responses in both mucosa and spleen, whereas subcutaneous (s.c.) immunization elicited only systemic CTLs (ref. 2). Such compartmentalization was also seen in mice by others<sup>17</sup>, but has not been demonstrated in primates. Intrarectally immunized mice were resistant to mucosal transmission of a recombinant vaccinia expressing HIV-1 gp160 (vPE16)<sup>26</sup>. Protection was CD8-dependent and required CTL in the mucosa<sup>6</sup>, suggesting that to prevent mucosal transmission, an AIDS vaccine would need to induce CTL in the mucosa itself.

Here we investigated whether these results would translate to retroviral infection in non-human primates. Mucosal and systemic immunization with the same AIDS vaccine have not been compared previously side-by-side in primates, and the role of gut mucosal CTL in clearing virus from this major SIV reservoir has not been examined. We again used a peptide vaccine to avoid viral vectors that might spread when comparing routes to determine the impact of CTL on a limited number of epitopes, and to





avoid inducing enhancing antibodies. We made analogous peptide vaccines, that had the same helper cluster peptide segments, but with several CTL epitopes from SIV Gag and Pol presented by the rhesus major histocompatibility (MHC) class I molecule, Mamu-A\*01 (refs. 18–20) (Fig. 1). The cluster helper peptides were broadly recognized by helper T cells from humans, mice and macaques<sup>3,10,12</sup> (and data not shown). All the animals except the controls received a mixture of all 4 peptides. As a less toxic adjuvant, we substituted for CT a mutant *E. coli* labile toxin LT(R192G)<sup>21,22</sup>, that we had found more effective in inducing a Th1 cytokine pattern and stronger CTL response<sup>7</sup>. For comparison with animals immunized intrarectally with peptides and LT(R192G) (group C), other animals were immunized s.c. with the peptides in Montanide ISA 51 (ref. 13) (group B), or intrarectally with LT(R192G) alone (group A) (Fig. 1). For intrarectal challenge, we chose simian/human immunodeficiency virus (SHIV)-Ku2 (refs. 23,24), a pathogenic strain expressing HIV-1 IIB gp160, including the helper epitopes, and SIV Gag and Pol, including the CTL epitopes.

**Induction of mucosal CTLs and helper T cells in primates**

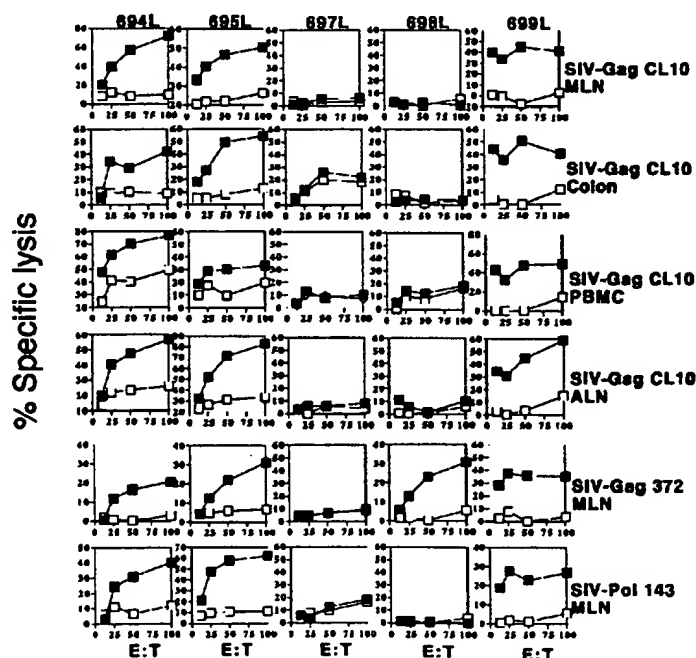
We first compared the CTLs and helper responses to the peptide vaccine delivered mucosally or s.c. after each immunization cycle (Fig. 1). After the first cycle, three of five intrarectally immunized macaques (group C) (694L, 698L, and 699L) had CTLs specific for the immunodominant Gag181 CL10 SIV (CTPYDINQML) epitope in mesenteric lymph nodes (MLN), and in two animals we obtained a large enough colonic biopsy to measure modest CTL responses in the colon (data not shown). All four s.c.-immunized macaques (group B) had substantial CL10-specific CTLs in the axillary lymph nodes (ALN), and two had these CTLs in peripheral blood mononuclear cells (PBMCs). Thus, one cycle of four intrarectal doses did not induce any higher a CTL response than four s.c. doses in the respective target sites of each immunization route.

However, after two cycles of four intrarectal vaccine doses, three of the five macaques (694L, 695L and 699L) made substantial Gag CL10 SIV-specific CTL responses in the MLN and colonic lamina propria as well as in systemic lymphoid tissues, that is, the ALN

and PBMCs (Fig. 2). These animals also had CTLs to the Pol and other Gag epitope in the MLN (Fig. 2) and similarly in ALN (data not shown). Although macaques 697L and 698L failed to manifest a response against Gag 181 CL10 in any tissue, macaque 698L did mount a strong CTL response in the MLN (Fig. 2) and ALN (data not shown) against the SIV Gag 372 epitope, which was also part of the vaccine. Overall, SIV-specific Mamu-A\*01-restricted CTLs were found both in mucosa and in systemic lymphoid tissues of four of five macaques given two cycles of intrarectal vaccine.

All four macaques (701L–704L) given two cycles of s.c. vaccine displayed substantial CL10 SIV Gag-specific CTLs in the ALN, and in the two macaques with the highest of these responses (701L and 702L), in PBMCs and the MLN as well (Fig. 3). These latter two macaques also responded to the Gag 372 and the Pol 143 epitopes in the ALN (Fig. 3). However, none of the control macaques (group A) exhibited CTL responses in any tissue (data not shown).

To further evaluate the CTL responses in immunized macaques, we quantified the number of CL10 Gag-specific CD8<sup>+</sup> T cells by stimulating *in vitro* with CL10 Gag-specific peptide and analyzing the CD8<sup>+</sup> CD3-gated T cells by flow cytometry using Mamu-A\*01/C9M tetramers. Although the number of tetramer-positive cells in freshly isolated tissues from immunized animals was not significantly greater than in control animals (data not



**Fig. 2** Induction of CTLs specific for SIV Gag CL10, Gag 372 or Pol 143, as indicated, in MLN, colonic lamina propria, PBMCs and ALN of group C macaques after 8 intrarectal immunizations with HIV/SIV peptide and LT(R192G). ■, lysis of targets with specific peptide; □, lysis of control targets without peptide. E:T, effector to target ratio.



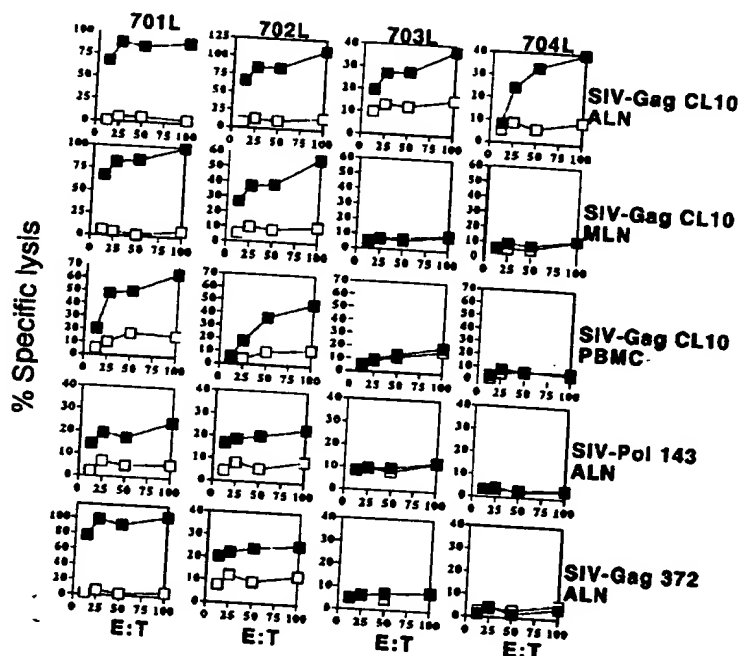


Fig. 3 Induction of CL10 SIV Gag-specific CTLs in ALN, MLN and PBMC and SIV Pol 143- and SIV Gag 372-specific CTLs in ALN of group B macaques after 8 s.c. immunizations with HIV/SIV peptide in Montanide ISA 51. ■, lysis of targets with specific peptide; □, lysis of control targets without peptide.

shown), macaque 694L of group C with the highest level of colonic CTLs showed a substantial number of tetramer-positive cells in stimulated cells from the colon (9%), MLN (2%) and ALN (9%). The rank of tetramer-positive cells generally correlated with that of CTLs. Similarly, in macaques 701L and 702L, with the highest CTL levels among s.c.-immunized macaques, tetramer-positive cell levels in the ALN were higher than in other group B macaques (17 and 25% versus 6 and 5%). Thus, tetramer staining and CTL lytic activity gave the same rank order of response for macaques in both the intrarectal- and s.c.-immunized groups.

To assess helper responses, we measured *in vitro* T-cell proliferation induced by the helper peptides in MLN or ALN cells after two vaccine cycles (Fig. 4a). Although the macaques were all selected for Mamu-A\*01, they were outbred for MHC class II and therefore heterogeneous in their helper T-cell response. In group C, the highest helper T-cell proliferative responses were noted in macaques 694L, 695L and 698L, which displayed the highest CTL responses, whereas macaque 697L, which exhibited no CTLs, showed only background proliferative responses (stimulation index ~1). Only in macaque 699L was a disparity between proliferation and cytolytic activity noted. A similar pattern was seen in group B, in that macaques 701L and 702L with the highest CTL responses also displayed the highest proliferative responses. This correlation between levels of vaccine-induced proliferative response and CTLs (Fig. 4b) ( $P < 0.002$ ), which has not been previously examined in a primate, suggests

that T-helper responses have an important role in the induction of optimal CTL responses in both mucosal and systemic tissues, and underscores the importance of this vaccine component.

Notably, a Mamu-A\*01-restricted CTL epitope has been identified in PCLUS3 (YAPPSGQI)<sup>25</sup>. We think it unlikely that CD8<sup>+</sup> T cells represent a major part of the proliferative response, because unless exogenous IL-2 is added, most of this response is from CD4<sup>+</sup> T cells. To confirm this interpretation, using antibodies to CD4 or CD8 and magnetic beads, we depleted the PBMCs of two representative animals that had remaining cells. The proliferative response was enriched in the CD8-depleted population and diminished in the CD4-depleted population, confirming that approximately 65–93% of the response was due to CD4<sup>+</sup> T cells (data not shown). In addition, after the second cycle, we detected no antibodies to PCLUS6.1 in any of the animals, nor to PCLUS3 in any of group C, but we detected low levels against PCLUS3 in three group B macaques (701L, 703L and 704L) (data not shown).

**Vaccine effect on intrarectal challenge with SHIV-Ku2**  
Eight weeks after the two cycles of peptide vaccine, all of the macaques were given an additional dose of vaccine plus adjuvant or adjuvant alone and then, two weeks later (week 25), were exposed intrarectally to approximately ten 50% animal infectious doses (AID<sub>50</sub>) of pathogenic SHIV-Ku2 (ref. 23,24) (one ill macaque was removed; see Methods). One macaque in each of groups A and C did not become viremic (as detected by the NASBA assay for viral RNA) whereas the remaining 9 macaques exhibited a peak plasma viral load between  $1 \times 10^5$ – $10^7$  mRNA copies per ml within 3 weeks following challenge exposure (Fig. 5). However, by the more sensitive PCR assay for proviral DNA in cells, we were able to detect infection in spleen cells in these two animals later at necropsy. As neither of these animals had a detectable immune response, it is unlikely that lack of viremia was due to an immune response, but probably represents a technical difficulty in obtaining reproducible infection in macaques by mucosal challenge. Therefore in addition to analyzing the data on the whole group, we have also examined the subset of animals that did become viremic, as only in the latter group could the effect of immunization on plasma viral load kinetics be followed.

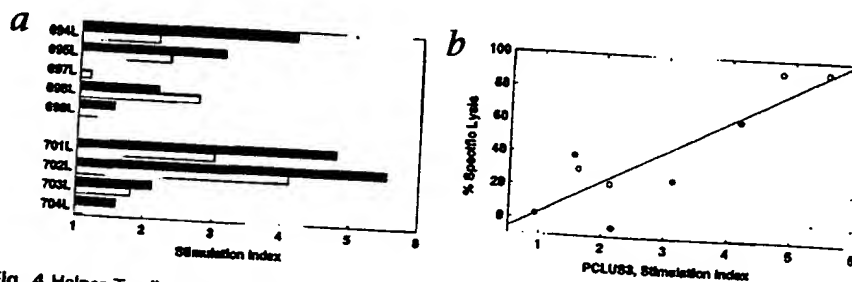
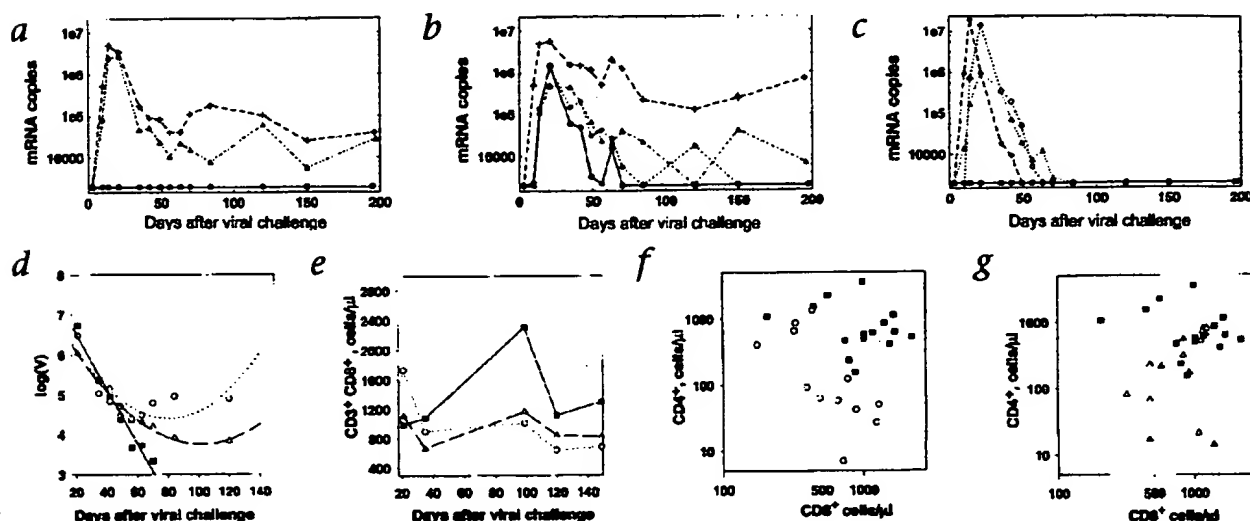


Fig. 4 Helper T-cell response correlates with CTL response. **a**, *In vitro* proliferative response against T-helper peptides PCLUS3 (■) and PCLUS6.1 (□) after 8 intrarectal (694L–699L) or s.c. (701L–704L) immunizations. The stimulation index was calculated as the ratio of thymidine incorporation into cellular DNA in wells with antigen over that in control wells with medium alone. **b**, Correlation of proliferative response to PCLUS3 with CTL response. ○, group C MLN; ●, group B ALN;  $r = 0.87$ ;  $P = 0.002$ .



**Fig. 5** Intrarectal and s.c. immunization of macaques with HIV/SIV peptide vaccine differentially protect against intrarectal challenge with pathogenic SHIV-Ku2. **a–c**, Viral load is expressed as viral RNA copies per ml plasma (by NASBA assay) versus time after challenge for macaque groups A–C. **a**, Group A, immunized intrarectally with LT(R192G) only. ●, 745L; +, 746L; △, 747L. **b**, Group B, immunized s.c. with HIV/SIV peptides in Montanide ISA51. △, 701L; ◆, 702L; ●, 703L; +, 704L. **c**, Group C, intrarectally immunized with the HIV/SIV peptides plus LT(R192G). ◆, 694L; △, 695L; ◇, 698L; ●, 697L. By a non-parametric repeated measures ANOVA, intrarectal group C viral titers were significantly lower than those of s.c. group B ( $P = 0.013$ ), the critical comparison, and showed a trend toward difference from those of group A ( $P = 0.14$ ), whereas s.c. group B was not significantly different from control group A ( $P = 0.92$ ). Examining only macaques with detectable viremia, as the kinetics of viral load could not be followed in the two others, the significance levels improved for group C versus A ( $P < 0.01$ ), and the differences remained significant for group C versus B ( $P = 0.031$ ) and not significant for group B

versus A ( $P = 0.65$ ). The overall comparison of A versus B versus C was significant ( $P = 0.017$ ). **d**, Typical time-course of mean viral load in the viremic subset macaques of groups A (circles), B (triangles), and C (squares), curve-fit as described in the Methods. Fit parameters: group A,  $b = 0.076$ ,  $c = 0.0005$ ; group B,  $b = 0.071$ ,  $c = 0.0003$ ; group C,  $b = 0.0693$ . Log(V) is  $\log_{10}$  of the viral load. **e**, Time-course of mean CD3<sup>+</sup>CD8<sup>+</sup> T lymphocyte counts per  $\mu$ l in the subsets of macaques in groups A (○), B (△), and C (■) that were actually viremic, parallel to **d**. **f** and **g**, At the several time-points during the steady-state phase of the infection process (35–176 d), PBMCs were analyzed by FACScan for the absolute number of CD3<sup>+</sup>CD8<sup>+</sup> or CD4<sup>+</sup> cells per ml, and plotted as a scatter diagrams comparing group C (■) with group A (○) in **f** or with group B (△) in **g**. In both cases, in the vertical dimension, group C exhibited larger numbers of CD3<sup>+</sup>CD8<sup>+</sup> cells than groups A or B ( $P = 0.005$  and  $P = 0.004$ , respectively, by the Mann–Whitney test). The absolute numbers of CD3<sup>+</sup>CD8<sup>+</sup> cells were higher in group C than group A ( $P = 0.01$ ), but not significantly different between group C and group B.

The outcome of SHIV infection in intrarectally immunized macaques (group C) differed substantially from the s.c. immunized (group B) or control macaques (group A). In the peak viral load, the differences between groups were not statistically significant (Fig. 5a–c). However, in all the group C macaques that became viremic, the viral load decreased substantially between days 20 and 70 after challenge, and the viral load reached a 'set point' below the level of detection (2000 mRNA copies/ml) before day 80. By contrast, in group B, one animal had a set point greater than  $1 \times 10^5$  copies per ml; two fluctuated between  $2 \times 10^3$  and  $3 \times 10^4$  copies per ml; and one fluctuated up to  $1 \times 10^4$  on day 63, but fell below the detection limit after day 70 (Fig. 5a–c).

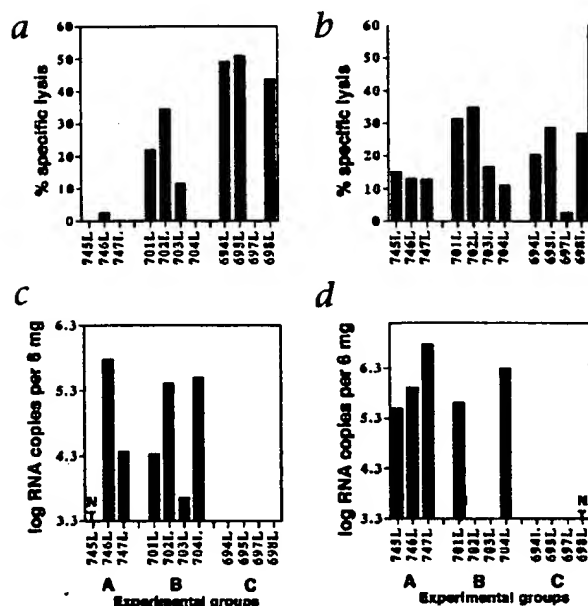
Statistical analysis showed that the concentration of SIV mRNA in the circulation dropped with similar half-lives ( $t_{1/2}$  of 10 days, 9.8 days and 9.2 days in groups C, B and A, respectively); however, in macaques immunized intrarectally, viral mRNA fell to an undetectable level by day 63, and remained undetectable subsequently (Fig. 5d). At day 63, early in the set-point, groups B and C were statistically different according to the Wilcoxon rank-sum test ( $P < 0.05$ ). Moreover, the viral loads at all time points measured during the steady-state or set-point phase (days 63–200) showed a significant difference between the intrarectal group C and the s.c. group B by a non-parametric repeated measures ANOVA<sup>26,27</sup> (see Fig. 5 legend). This comparison remained statistically significant even when the number of

time-points in the analysis was reduced. Thus, despite small sample sizes, these results suggest that mucosal immunization was more effective than s.c. immunization.

In addition to plasma viral load, we also assessed the number of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and clinical course. The former was assessed by plotting scatter plots of the absolute number of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in all peripheral blood specimens taken at various times starting on day 35 after viral challenge in all viremic macaques. By inspection, CD4 counts for group C were mostly higher than those for group A (Fig. 5f;  $P = 0.005$ ) or group B (Fig. 5g;  $P = 0.004$ ). Although CD8<sup>+</sup> T-cell counts differed less (horizontal axis), some expansion of CD8<sup>+</sup> T cells was noted in a kinetic plot (Fig. 5e). Clinically, in each of groups A and B the animal with the highest viral load developed evidence of opportunistic infection (746L, gingivitis and pneumonia; 704L, severe gingivitis, blepharitis, facial dermatitis and pathologic evidence of pulmonary pneumocystis). None of the animals in group C showed evidence of AIDS-like illness.

Two hundred days after SHIV-Ku2 virus challenge, we killed the macaques and determined CTL responses in colon and ALN, and viral loads in gut tissue. We could measure CTL activity in freshly obtained cells without stimulation *in vitro*, as has been seen in HIV infection<sup>28</sup>. Notably, compared with control target background, intrarectally immunized group C macaques that exhibited viral set-points below the level of detection (694L, 695L

## ARTICLES



**Fig. 6** Intrarectal peptide vaccine was more effective than s.c. peptide vaccine at priming colonic CTLs for a secondary response induced by challenge infection with SHIV, and clearing virus from the gut. Intestinal tissue and ALN were obtained at the time of necropsy, 200 d after the challenge with pathogenic SHIV-ku2. **a** and **b**, Freshly isolated lymphocytes from colonic lamina propria (**a**) and ALN (**b**) were used as effector cells, without restimulation *in vitro*, at an effector-to-target ratio of 100:1. Results show specific lysis of peptide-pulsed targets less than that of control unpulsed targets. **c** and **d**, Viral RNA load in the colon (**c**) and jejunum (**d**) were determined on tissues obtained at the same time-point. The lower limit of detection was 2000 copies per 6 mg tissue, corresponding to a log<sub>10</sub> value of 3.3 on the ordinate. In the colon (**c**), although the numbers are small, group C differs significantly by the  $\chi^2$  median test from group A ( $P = 0.014$ ) and from group B ( $P = 0.005$ ). In the jejunum (**d**), group C is different from A ( $P = 0.008$ ) and from group B ( $P = 0.05$ ). In all cases, groups A and B are not significantly different from each other.

and 698L) consistently exhibited substantially higher CL10 SIV Gag-specific CTL activity in the colon than s.c. immunized group B macaques (Fig. 6a), and control group A had almost none. In contrast, while CTL levels in ALN were also increased after viral challenge, in this case the increase in both groups was equivalent. In both cases, macaques that made a strong CTL response after vaccination were the ones that showed activity *ex vivo* 200 days after viral infection. These results suggest that the vaccine-primed CTL memory cells were reactivated by the SHIV infection, as recently reported for a DNA vaccine<sup>29</sup>.

These data suggest an explanation for the lower plasma set-points in the intrarectally immunized macaques as the gut mucosa is a major site of viral replication<sup>1</sup>; we therefore hypothesized that higher levels of CTLs at this site might lead to better control of infection. Such control of virus in the gut mucosa has not been previously examined. To test this new hypothesis, we measured virus levels in the colon and jejunum at the time of necropsy, 200 days after challenge (Fig. 6c and d). Notably, none of the group C animals had detectable virus (< 2000 copies) in colon or jejunum, whereas the group A and B animals all had levels 10–100-fold over the detection limit (except for animal 703L, that also had the lowest plasma viral load in group B). Thus, the difference in viral load in the major gut reservoir is even more

pronounced than the difference in viremia. Group C was significantly different from groups B and A (see figure legend). In the colon, virus levels were largely inversely related to CTL levels (Fig. 6a and c). These results indicate that the better control of plasma viremia in the intrarectally immunized macaques was mediated by a higher level of CTLs in the gut. Virus was more effectively cleared from this major site of replication, which seeds the blood stream. This key role of gut mucosal CTLs in control of HIV/SIV infection has not been previously recognized.

### Discussion

In these studies we demonstrated by several independent criteria that intrarectally administered synthetic multipeptide HIV/SIV peptide vaccine with mutant LT (R192G) as an adjuvant induces a mucosal CTL response. This novel vaccine provided better protection against intrarectal SHIV infection than a s.c.-administered vaccine comprising the same peptides that induce as high or higher systemic CTL responses. Reduction of viremia correlated with preservation of CD4<sup>+</sup> T-cell counts, with post-infection CTL activity in the gut and with clearance of SHIV from the gut mucosal tissues. These data represent the first evidence from non-human primates for the concept that HIV/SIV-specific CTL responses in the mucosa are at least as important as systemic CTLs in protection against HIV/SIV infection. This is not only because of the importance of mucosal transmission, but also because the gut is a major reservoir of virus. This fundamental concept will be important for the optimal design of effective AIDS vaccines.

Neither of the immunization regimens led to CTL responses sufficient to reduce initial viral expansion and dissemination. Nevertheless, intrarectal immunization with the peptide vaccine did have a positive impact on infection during the plateau phase, in that the set point actually dropped below the level of detection. The difference in viremia between intrarectal and s.c. groups was significant whether or not one included the two animals that never became viremic, although without these two, the smaller number of animals reduced the power of the test and made the difference, although still significant, statistically less robust. Because these animals had no detectable immune response, their lack of viremia is likely due to inadequate intrarectal challenge, and in any case, prevented study of the kinetics of their viremia.

Intrarectal immunization also led to greater preservation of CD4<sup>+</sup> T cells than s.c. immunization. This is another measure of infection outcome, independent of viral load, indicating that intrarectal immunization is superior to s.c. immunization in protection against intrarectal infection.

We hypothesize that rectal immunization with peptide vaccine was able to greatly reduce viral replication in the main site of viral burden, the mucosal tissues, because mucosal immunization induced mucosal memory CTLs. Indeed, protection correlated best with CTLs present in the colon after challenge, and the difference between intrarectal and s.c. immunization in reducing viral burden in the gut was even more pronounced than that in plasma (Fig. 6). A recent study of a systemically administered DNA vaccine showed a similar reduction in plasma viral set-point, but only when given with a chimeric interleukin-2/immunoglobulin construct<sup>29</sup>. Mucosal immunization may achieve the result without cytokine amplification because it focuses the CTLs in the major site of viral replication. Other studies have shown that animals previously infected with SIV, which is known to infect the gut and could induce mucosal immunization, were protected

against mucosal transmission of SIV (refs. 8,30). Murphey-Corb *et al.*<sup>8</sup> found that during SIV infection, protection correlated in some animals with CTLs in the jejunum. Targeted iliac lymph node immunization has also protected against mucosal SIV infection (ref. 4). A recent study of DNA/modified vaccinia Ankara (MVA) prime-boost strategy also showed reduction of viral load<sup>31</sup>. Here we establish the importance of mucosal immunization by a direct comparison of mucosal and systemic immunization against SIV/HIV in a primate, and by examining the effect on viral levels in the gut reservoir.

Such focusing of CTLs in the gut tissues where HIV and SIV replicate depends not only on immunization through a mucosal route, as we have done, but also on the existence of a compartmentalized mucosal CTL response. We<sup>2,32</sup> and others<sup>17</sup> have seen this compartmentalization in mice, but not in primates. This asymmetry in trafficking suggests that mucosal immunization, which can induce CTLs in both compartments (in contrast to systemic immunization), may be effective at eliminating both systemic as well as mucosal virus. Our results also suggest that this compartmentalization applies to non-human primates, although definitive proof is lacking because we could not obtain colonic biopsies from the s.c.-immunized macaques before challenge. Furthermore, post-challenge CTL responses in the colon were higher in the intrarectally immunized group than the s.c. group. Because the control-infected animals had almost no specific colonic CTLs, such CTLs likely represent in part a secondary response induced by infection but dependent on priming by the vaccine. Thus, these data provide further evidence for more effective priming of gut CTLs by mucosal immunization.

It is becoming clear that the outcome of SIV/HIV infection depends on the interplay of the CD4<sup>+</sup> T-helper function and the preservation of CD8<sup>+</sup> CTL function<sup>33</sup>. With regard to the need for helper T-cell induction by vaccines, we and others have demonstrated the importance of a linked helper epitope for CTL induction in mice<sup>34,35</sup>, but the requirement for help to induce CTLs is not known in primates. Thus, our results are important in showing that SIV-specific CTL activity correlated with the HIV helper epitope-specific T-cell proliferative activity, not previously reported in a primate.

We are not convinced that we have optimized mucosal immunization. We used mutant *E. coli* labile toxin LT(R1926)<sup>22</sup> as an alternative adjuvant to the more usual CT, because it is less toxic and does not inhibit endogenous interleukin-12 production as does CT (refs. 7,37). It is also apparent that we had to vary the adjuvant at the same time we varied the immunization route. A recent study by Allen *et al.*<sup>36</sup> showed that CTLs specific for a single TAT epitope can select for new viral variants within two months of infection. Our data show that even a synthetic vaccine with only two helper and three CTL epitope peptides, given mucosally, can impact the course of pathogenic SHIV infection. A mucosal vaccine that incorporates more epitopes and immuno-enhancing molecules may be even more effective at controlling virus infection by reducing the viral reservoir in the gut, independent of the route of exposure.

## Methods

**Macaques and vaccines.** Indian rhesus macaques (*Macaca mulatta*) were matched for genetic origin, source, and comparable age and weight, and maintained in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International and under the approval of the Applied BioScience Labs Animal Center Review Committee. All were seronegative for SIV, simian retroviruses 1, 2 and 5, and simian T-cell leukemia/lymphotropic virus type 1 (STLV-1) prior to the study, and

were Mamu-A\*01-positive MHC type as determined by PCR-sequence-specific primers and direct sequencing<sup>38</sup>. The peptides in Fig. 1 each contain an HIV-envelope helper epitope<sup>10</sup> and an SIV Gag<sup>19</sup> or Pol<sup>20</sup> CTL epitope.

**SHIV-Ku2 virus stock.** SHIV-Ku2 is a chimeric virus containing the HIV-1 IIIB strain (HXBc2) envelope gene and SIVmac239 gag and pol genes, and is pathogenic in rhesus macaques<sup>23,24</sup>. SHIV-Ku2 stock was isolated from PBMC of SHIV-Ku2-infected macaques and titered *in vivo* intrarectally using 10-fold serial dilutions. Inoculation with 1 ml undiluted virus infected 8/8 (100%), as shown by the SIV plasma RNA within 2 weeks after virus challenge. Virus stock diluted 1:10 infected 2/4 macaques (50%), and none was infected with dilutions  $\geq 1:100$ . Thus, the undiluted virus challenge dose contained  $\sim 10$  AID<sub>50</sub> for intrarectal administration.

**Immunization and challenge of macaques.** Each intrarectal peptide vaccine dose contained 0.5 mg of each peptide (total 2 mg peptide) mixed with mutant *Escherichia coli* labile toxin LT(R192G) as mucosal adjuvant (50  $\mu$ g per immunization)<sup>22</sup>. Each s.c. vaccine dose was administered in the upper arm with the same peptide mixture emulsified 1:1 with Montanide ISA 51 adjuvant (Seppic, Fairfield, New Jersey). Control macaques received intrarectal LT(R192G) alone (50  $\mu$ g per dose) by the same schedule (Fig. 1). For intrarectal inoculations, animals were sedated with ketamine hydrochloride (10 mg/kg i.m.) and placed within a biosafety cabinet in ventral recumbency with the hindquarters elevated. The tail was elevated dorsally and a 3-cc slip-tip syringe was atraumatically inserted into the rectum. Vaccine or virus was administered and the tail lowered assuring complete delivery.

Before challenge, one animal, #699L, that had received the intrarectal vaccine (group C), developed peritonitis following laparotomy for the final colonic biopsy, requiring urgent surgery to remove a large segment of devitalized bowel, and nearly died. Thus, shortly prior to challenge with SHIV-Ku2 the immune system of this macaque was under considerable stress, as corroborated by greatly decreased numbers of circulating lymphocytes (including CD8<sup>+</sup> T cells), monocytes and neutrophils immediately prior to challenge. Although challenged, this animal could not be included in the analysis because of the apparent immune deficiency related to the severe peritonitis.

**Biopsies and isolation of lamina propria lymphocytes.** MLN and intestinal tissue were obtained by laparotomy. MLN were chosen as an interface between intestinal and systemic immune systems, and para-aortic and para-iliac nodes were not accessible in this procedure. Intestinal tissue was obtained either from colonic-wedge resection ( $\sim 4$  cm<sup>2</sup>) at laparotomy, or at the time of necropsy ( $\sim 100$  cm<sup>2</sup>). Lymphocytes were prepared as previously established for human tissue<sup>40</sup>.

**Lymphocyte proliferation assay.** Lymphocytes from blood and lymph nodes were freshly isolated by density-gradient centrifugation on Ficoll, resuspended in complete T-cell medium (CTM)<sup>41</sup>, and cultured at  $3 \times 10^5$  cells per well in 96-well plates for 3 d in the absence or presence of T-helper peptides (PCLUS3 or PCLUS6.1)<sup>10,41</sup>, using PHA as a positive control, and then pulsed for 8 h with 1 mCi [<sup>3</sup>H]thymidine.

**CTL and tetramer assays.** Immune cells were cultured 7 d at  $5 \times 10^4$  per ml in 12-well plates with separate synthetic SIV CTL epitope peptides in CTM. On day 3 rIL-2 (Boehringer) (20 international units per ml) was added. Autologous B-lymphoblastoid cell line (B-LCL) target cells were pulsed with 1  $\mu$ M peptide for 2 h during <sup>51</sup>Cr labeling, and cultured with effectors at 37 °C for 4 h. Specific release was calculated as described<sup>42</sup>. Spontaneous release was  $< 15\%$  of maximal release in all assays. Soluble tetrameric Mamu-A\*01/CM9 complex conjugated to PE-labeled streptavidin prepared as described<sup>19,42</sup> was used to stain CD8<sup>+</sup> cells gated on CD3<sup>+</sup> cells (as CD8<sup>+</sup>-specific monoclonal antibodies can bind to natural killer cells of rhesus macaques<sup>43</sup>) and analyzed on FACScan (Becton-Dickinson).

**Determination of viral load.** SIV<sub>mac</sub>251 mRNA in plasma was quantified by nucleic acid sequence-based amplification (ref. 44), with a detection limit of  $2 \times 10^3$  RNA copies. For colon and jejunum, snap-frozen samples of 300–500 mg were homogenized and lysed in 1 ml of lysis buffer. 20- $\mu$ l samples were analyzed, and the results normalized to 6 mg tissue.

## ARTICLES

For detection of virus in spleen cells at necropsy, thawed cells were washed in medium with 10% FCS, centrifuged at 10,000g for 15 min, lysed in DNA-STAT (Tel-Test, Friendswood, Texas), and processed according to the directions. The DNA was resuspended in 10 mM Tris and 0.1 mM EDTA, and amplified in 50 µl PCR buffer, 2 µM primers, and 200 µM NTPs, for 25 cycles of 95 °C (30 s), 50 °C (30 s) and 72 °C (3 min). The amplified product was run on a 2% agarose gel. The sensitivity was about 1000 copies of DNA per sample.

**Software for fitting and statistical analysis.** The kinetics of plasma viral mRNA during the 'decay stage' (21 d after challenge) were analyzed by an exponential function:  $V(t) = V_0 e^{-ct}$ , where  $V_0$  is the maximum number of mRNAs extrapolated on day 0,  $b$  is the rate of mRNA decline in plasma, and  $t$  is time (days). Alternatively we used the model,  $V(t) = V_0 e^{-ct} + a e^{-ct}$  (where  $c$  is the non-negative constant), which assumes a 'virus-escape' mechanism. Curve fitting and statistical analysis were performed in MLAB (Chilized Software, Silver Spring, Maryland), and parameters estimated using the Marquardt-Levenberg method. Statistica-6 (StatSoft, Tulsa, Oklahoma) and SigmaPlot-2000 (SSPS, Chicago, Illinois) were also employed. Statistical comparisons were performed using the Mann-Whitney, Wilcoxon, Kruskal-Wallis and  $\chi^2$  tests, as well as a non-parametric repeated measures ANOVA for plasma viral loads over time<sup>26,27</sup> because the data could not be taken as normally distributed. We found no significant interaction between time and treatment by this repeated measures ANOVA, consistent with the initial assumption of lack of interaction between time and treatment. Software for the non-parametric repeated measures ANOVA is available from V. Kuznetsov ([vk28u@nih.gov](mailto:vk28u@nih.gov)).

## Acknowledgments

We thank M. Spano and the animal care staff at the ABL for their assistance; R. Pal for the titrated stock of SHIV-Ku2; J.-P. Planchot for the Montanide ISA51; M. Lewis for the DNA PCR of provirus; V. Stepanov, Y. Tsyurin, S. Steinberg, D. Venzon and E. Brunner for assistance, advice and critical comments on statistical analysis; V. Hirsch, B. Moss, and J. Snyder for critical reading of the manuscript and helpful suggestions; and N. Miller for support of the animals. The work of A.S. was partially supported by NIH contract N01-AI-95362 and grant R24 RR 15731.

RECEIVED 17 SEPTEMBER; ACCEPTED 30 OCTOBER 2001

1. Veazey, R.S. et al. Gastrointestinal tract as a major site of CD4<sup>+</sup> T cell depletion and viral replication in SIV infection. *Science* **280**, 427–431 (1998).
2. Belyakov, I.M. et al. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. *Proc. Natl. Acad. Sci. USA* **95**, 1709–1714 (1998).
3. Berzofsky, J.A. et al. Approaches to improve engineered vaccines for human immunodeficiency virus (HIV) and other viruses that cause chronic infections. *Immunol. Rev.* **170**, 151–172 (1999).
4. Lehner, T., Bergmeier, L., Wang, Y., Tao, L. & Mitchell, E. A rational basis for mucosal vaccination against HIV infection. *Immunol. Rev.* **170**, 183–196 (1999).
5. Czerkinsky, C. et al. Mucosal immunity and tolerance: relevance to vaccine development. *Immunol. Rev.* **170**, 197–222 (1999).
6. Belyakov, I.M. et al. The importance of local mucosal HIV-specific CD8<sup>+</sup> cytotoxic T lymphocytes for resistance to mucosal-viral transmission in mice and enhancement of resistance by local administration of IL-12. *J. Clin. Invest.* **102**, 2072–2081 (1998).
7. Belyakov, I.M., Ahlers, J.D., Clements, J.D., Strober, W. & Berzofsky, J.A. Interplay of cytokines and adjuvants in the regulation of mucosal and systemic HIV-specific cytotoxic T lymphocytes. *J. Immunol.* **165**, 6454–6462 (2000).
8. Murphy-Corb, M. et al. Selective induction of protective MHC class I restricted CTL in the intestinal lamina propria of rhesus monkeys by transient SIV infection of the colonic mucosa. *J. Immunol.* **162**, 540–549 (1999).
9. Kaul, R. et al. HIV-1-specific mucosal CD8<sup>+</sup> lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi. *J. Immunol.* **164**, 1602–1611 (2000).
10. Berzofsky, J.A. et al. Construction of peptides encompassing multideterminant clusters of HIV envelope to induce *in vitro* T-cell responses in mice and humans of multiple MHC types. *J. Clin. Invest.* **88**, 876–884 (1991).
11. Takahashi, H. et al. An immunodominant epitope of the HIV gp160 envelope glycoprotein recognized by class I MHC molecule-restricted murine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **85**, 3105–3109 (1988).
12. Ahlers, J.D. et al. Construction of an HIV-1 peptide vaccine containing a multideterminant helper peptide linked to a V3 loop peptide 18 inducing strong neutralizing antibody responses in mice of multiple MHC haplotypes after two immunizations. *J. Immunol.* **150**, 5647–5665 (1993).
13. Ahlers, J.D. et al. Candidate HIV type 1 multideterminant cluster peptide-P18MN vaccine constructs elicit type 1 helper T cells, cytotoxic T cells, and neutralizing antibody, all using the same adjuvant immunization. *AIDS Res. Hum. Retroviruses* **12**, 259–272 (1996).
14. Palker, T.J. et al. Polyvalent human immunodeficiency virus synthetic immunogen comprised of envelope gp120 T helper cell sites and B cell neutralization epitopes. *J. Immunol.* **142**, 3612–3619 (1989).
15. Hart, M.K. et al. Priming of anti-human immunodeficiency virus (HIV) CD8<sup>+</sup> cytotoxic T cells *in vivo* by carrier-free HIV synthetic peptides. *Proc. Natl. Acad. Sci. USA* **88**, 9448–9452 (1991).
16. Yasutomi, Y., Palker, T.J., Gardner, M.B., Haynes, B.F. & Letvin, N.L. Synthetic peptide in mineral oil adjuvant elicits simian immunodeficiency virus-specific CD8<sup>+</sup> cytotoxic T lymphocytes in Rhesus monkeys. *J. Immunol.* **151**, 5096–5105 (1993).
17. Rose, J.R., Williams, M.B., Rott, L.S., Butcher, E.C. & Greenberg, H.B. Expression of the mucosal homing receptor  $\alpha 4 \beta 7$  correlates with the ability of CD8<sup>+</sup> memory T cells to clear rotavirus infection. *J. Virol.* **72**, 726–730 (1998).
18. Miller, M.D., Yamamoto, H., Hughes, A.L., Watkins, D.I. & Letvin, N.L. Definition of an epitope and MHC class I molecule recognized by gag-specific cytotoxic T lymphocytes in SIVmac-infected rhesus monkeys. *J. Immunol.* **147**, 320–329 (1991).
19. Kuroda, M.J. et al. Analysis of Gag-specific cytotoxic T lymphocytes in simian immunodeficiency virus-infected rhesus monkeys by cell staining with a tetrameric major histocompatibility complex class I-peptide complex. *J. Exp. Med.* **187**, 1373–1381 (1998).
20. Allen, T.M. et al. CD8<sup>+</sup> lymphocytes from simian immunodeficiency virus-infected rhesus macaques recognize 14 different epitopes bound by the major histocompatibility complex class I molecule mamu-A\*01: implications for vaccine design and testing. *J. Virol.* **75**, 738–749 (2000).
21. Dickinson, B.L. & Clements, J.D. Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvant activity from ADP-ribosyltransferase activity. *Infect. Immun.* **63**, 1617–1623 (1995).
22. Morris, C.B., Cheng, E., Thanawastien, A., Cardenas-Freytag, L. & Clements, J.D. Effectiveness of intranasal immunization with HIV-gp160 Env CTL epitope peptide (E7) in combination with the mucosal adjuvant LT(R192C). *Vaccine* **18**, 1944–1951 (2000).
23. Joag, S.V. et al. Chimeric simian/human immunodeficiency virus that causes progressive loss of CD4<sup>+</sup> T cells and AIDS in pig-tailed macaques. *J. Virol.* **70**, 3189–3197 (1996).
24. Joag, S.V. et al. Chimeric SHIV that causes CD4<sup>+</sup> T cell loss and AIDS in rhesus macaques. *J. Med. Primatol.* **27**, 59–64 (1998).
25. Egan, M.A. et al. Use of major histocompatibility complex class I-peptide/β2M tetramers to quantitate CD8<sup>+</sup> cytotoxic T lymphocytes specific for dominant and non-dominant viral epitopes in simian-human immunodeficiency virus-infected rhesus monkeys. *J. Virol.* **73**, 5466–5472 (1999).
26. Brunner, E., Munzel, U. & Purl, M.L. Ranks-score tests in factorial designs with repeated measures. *J. Multivariate Anal.* **70**, 286–317 (1999).
27. Brunner, E. & Purl, M.L. Nonparametric methods in factorial designs. *Statistical Papers* **42**, 1–52 (2001).
28. Walker, B.D. et al. HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature* **328**, 345–351 (1987).
29. Barouch, D.H. et al. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290**, 486–492 (2000).
30. Cranage, M.P. et al. Intrarectal challenge of macaques vaccinated with formalin-inactivated simian immunodeficiency virus. *Lancet* **339**, 273–274 (1992).
31. Amara, R.R. et al. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* **292**, 69–74 (2001).
32. Belyakov, I.M. et al. Induction of mucosal CTL response by intrarectal immunization with a replication-deficient recombinant vaccinia virus expressing HIV 89.6 envelope protein. *J. Virol.* **72**, 8264–8272 (1998).
33. Rosenberg, E.S. et al. Vigorous HIV-1-specific CD4<sup>+</sup> T cell responses associated with control of viremia. *Science* **278**, 1447–1450 (1997).
34. Shirai, M. et al. Helper-CTL determinant linkage required for priming of anti-HIV CD8<sup>+</sup> CTL *in vivo* with peptide vaccine constructs. *J. Immunol.* **152**, 549–556 (1994).
35. Ahlers, J.D., Takeshita, T., Pendleton, C.D. & Berzofsky, J.A. Enhanced immunogenicity of HIV-1 vaccine construct by modification of the native peptide sequence. *Proc. Natl. Acad. Sci. USA* **94**, 10856–10861 (1997).
36. Ossendorp, F., Mengede, E., Camps, M., Rikun, R. & Melief, C.J. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J. Exp. Med.* **187**, 693–702 (1998).
37. Braun, M.C., He, J., Wu, C.-Y. & Kelsall, B.L. Cholera toxin suppresses interleukin (IL)-12 production and IL-12 receptor B1 and B2 chain expression. *J. Exp. Med.* **189**, 541–552 (1999).
38. Allen, T.M. et al. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature* **407**, 386–390 (2000).
39. Krapp, L.A., Lehmann, E., Piekarczyk, M.S., Urvater, J.A. & Watkins, D.I. A high frequency of Mamu-A\*01 in the rhesus macaque detected by polymerase chain reaction with sequence-specific primers and direct sequencing. *Tissue Antigens* **50**, 657–661 (1997).
40. Bull, D.M. & Bookman, M.A. Isolation and functional characterization of human intestinal mucosal lymphoid cells. *J. Clin. Invest.* **59**, 966–974 (1977).
41. Hosmalin, A. et al. Priming with helper T-cell epitope peptides enhances the antibody response to the envelope glycoprotein of HIV 1 in primates. *J. Immunol.* **146**, 1667–1673 (1991).
42. Altman, J.D. et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**, 94–96 (1996).
43. Kuroda, M.J. et al. Emergence of CTL coincides with clearance of virus during primary simian immunodeficiency virus infection in rhesus monkeys. *J. Immunol.* **162**, 5127–5133 (1999).
44. Romano, J.W. et al. Quantitative evaluation of simian immunodeficiency virus infection using NASBA technology. *J. Virol. Methods* **86**, 61–70 (2000).